UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
Instituto de Ciências Básicas da Saúde
Departamento de Fisiologia
Programa de Pós-Graduação em Ciências Biológicas: Fisiologia

INFLUÊNCIA DO ESTRESSE OXIDATIVO NO DESENVOLVIMENTO DA HIPERTROFIA E INSUFICIÊNCIA CARDÍACA INDUZIDA PELO HIPERTIREOIDISMO EM RATOS

ALEX SANDER DA ROSA ARAUJO

Orientadora: Dra. ADRIANE BELLÓ KLEIN
Co-orientadora: Dra. MARIA FLÁVIA MARQUES RIBEIRO

Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas, área de concentração: Fisiologia, Universidade Federal do Rio Grande do Sul, como requisito parcial para a obtenção do grau de Doutor em Ciências Biológicas - Fisiologia

Porto Alegre, 2006

AGRADECIMENTOS

A Deus por me carregar nos braços durante todos os momentos da minha vida.

A minha orientadora Adriane Belló-Klein por confiar no meu trabalho.

A minha co-orientadora Maria Flávia Marques Ribeiro pelo grande apoio ao longo da tese.

À professora Susana Llesuy pela dedicação.

À Tânia Regina Gatteli pela ajuda na realização dos experimentos.

A todos os demais colegas do laboratório pelo incentivo ao trabalho.

Ao Laboratório de Análises Clínicas Weimann pela colaboração e pelo empenho em prol da ciência.

A minha namorada amada Fernanda pelo auxílio, compreensão e paciência.

Aos meus pais por todo amor e carinho.

ÍNDICE

LISTA DE FIGURAS ---------- *V*

LISTA DE ABREVIATURAS ---------- *VI*

RESUMO ---------- *VIII*

1.INTRODUÇÃO ---------- *1*

1.1. Hipertireoidismo ---------- **1**

1.2. Hipertireoidismo e Estresse Oxidativo ---------- **2**

1.3. Espécies Ativas de Oxigênio (EAO), Sistemas de Defesa Antioxidante e Estresse Oxidativo ---------- **4**

1.4. EAO no processo de sinalização celular ---------- **10**

1.5. Hipertrofia Cardíaca, Sinalização Intracelular e Hipertireoidismo ---------- **14**

2. HIPÓTESE ---------- *16*

3 .OBJETIVOS ---------- *17*

Objetivo Geral ---------- **17**

Objetivos Específicos ---------- **17**

4. METODOLOGIA ---------- *19*

4.1. Animais ---------- **19**

4.2. Indução do Hipertireoidismo ---------- **19**

4.3. Grupos e Protocolos Experimentais ---------- **20**

4.3.1. Artigo 1- Myocardial Antioxidant Enzyme Activities and Concentration and Glutathione Metabolism in Experimental Hyperthyroidism *(Molecular and Cellular Endocrinology 249 (2006) 133-139)* ---------- 20

4.3.2. Artigo 2: Oxidative stress activates insulin-like growth factor I receptor protein expression mediating cardiac hypertrophy induced by thyroxine (*submetido em 2006 para Molecular and Cellular Biochemistry, aguardando o processo de revisão*) ---------- 20

4.3.3. Artigo 3: Oxidative stress role in the AKT pathway activation in experimental hyperthyroidism (*submetido em 2006 para Journal Molecular Cardiology, aguardando o processo de revisão*) ---------- 21

4.4. Protocolo Experimental ---------- **21**

4.5. Dosagem Hormonal ---------- **22**

4.6. Avaliações Hemodinâmicas e Cálculo do Índice de Hipertrofia Cardíaca ---------- **23**

4.7. Preparo dos Homogeneizados ---------- **24**

4.8. Quantificação de Proteínas ---------- **24**

4.9. Medidas de dano oxidativo ---------- **25**

4.10. Determinação da Concentração da Glutationa Total e Reduzida ---------- **26**

4.11. Capacidade Antioxidante Total (TRAP) ---------- **26**

4.12. Atividade das enzimas antioxidantes ---------- **27**

4.13. Análise de nitritos e nitratos ---------- **29**

4.14. Medida do peróxido de hidrogênio ---------- **29**

4.15. Análise por "Western blot" ---------- **30**

5. ANÁLISE ESTATÍSTICA ---------- ***32***

6. RESULTADOS ---------- ***33***

6.1 Artigo 1: ***Myocardial Antioxidant Enzyme Activities and Concentration and Glutathione in Experimental Hyperthyroidism (Molecular and Cellular Endocrinology 249: 133-139, 2006).*** ---------- **34**

6.2 Artigo 2*: Oxidative stress activates insulin-like growth factor I receptor protein expression mediating cardiac hypertrophy induced by thyroxine (Submetido para: Molecular and Cellular Biochemistry)* ---------- **42**

6.3. Artigo 3: ***Oxidative stress role in the AKT pathway activation in experimental hyperthyroidism (Journal of Molecular and Cellular Cardiology)*** ---------- **69**

7. DISCUSSÃO CONCLUSIVA ---------- ***99***

8. PERSPECTIVAS ---------- ***105***

9. REFERÊNCIAS BIBLIOGRÁFICAS ---------- ***107***

LISTA DE FIGURAS

Figura 1: Estrutura química do α-tocoferol e do ϒ-tocoferol----------8

Figura 2: Esquema proposto para o desenvolvimento de hipertrofia cardíaca induzida pela ativação da MAPK ----------13

Figura 3: Diferentes vias de sinalização intracelulares envolvidas no desenvolvimento da hipertrofia cardíaca ----------15

LISTA DE ABREVIATURAS

AKT: proteína quinase B
AMPc: adenosina 3,5 monofostato cíclico
CAT: catalase
EAO: espécies ativas de oxigênio
EAN: espécies ativas de nitrogênio
EDRF: fator relaxante derivado do endotélio
EGF: fator de crescimento epidermal
ERK: quinase regulada por sinal extracelular
FAD: Flavina adenina dinucleotídeo
FGF: fator de crescimento de fibroblasto
GPx: glutationa peroxidase
GR: glutationa redutase
GSH: glutationa reduzida
GSK-3β: glicogênio sintase quinase-3 beta
GSSG: dissulfeto de glutationa
GST: glutationa S transferase
IGF-I: fator de crescimento *insulin like*-I
IGF-IR: receptor do fator de crescimento *insulin like*-I
JNK: c-jun-NH_2 terminal quinase
LPO: lipoperoxidação
PDFVE: pressão diastólica final do ventrículo esquerdo
PSVE: pressão sistólica ventricular esquerda
MAPK: membros da família das proteínas quinases ativadas por mitógenos
NO: óxido nítrico
NOS: óxido nítrico sintase
PDGF: fator de crescimento derivado de plaquetas
PI3K: fosfatidilinositol 3 quinase
QL: quimiluminescência

RTK: receptor tirosina quinase

SOD: superóxido dismutase

TBARS: substâncias reativas ao ácido tiobarbitúrico

TRAP: capacidade antioxidante total

TSH: hormônio estimulate da tireóide

T3: triiodotironina

T4: tiroxina

RESUMO

A elevação do metabolismo basal e do consumo de oxigênio produzido pelos hormônios da tireóide podem predispor a célula a produzir espécies ativas de oxigênio (EAO). Dentre as alterações morfométricas causadas pela disfunção da tireóide, destaca-se a hipertrofia cardíaca. Este crescimento da massa do coração pode ser creditado à ação genômica dos hormônios tireoidianos. Entretanto, o desenvolvimento da hipertrofia cardíaca pode estar correlacionado com as EAO. Por isso, objetivou-se estudar não somente a relação das EAO com desenvolvimento da hipertrofia e a insuficiência cardíaca no hipertireoidismo, mas também os mecanismos moleculares deste processo, utilizando tratamento com um antioxidante clássico, a vitamina E. Foram usados ratos Wistar divididos em quatro grupos (n=10): controle, vitamina E, hipertireoideo, hipertireoideo+vitamina E. O hipertireoidismo foi desenvolvido através da administração de L–tiroxina (12mg/L na água de beber, durante 28 dias). A vitamina E foi administrada (20mg/kg/dia i.p.) por 28 dias. A medida da massa cardíaca foi avaliada pela razão do peso do coração pelo peso corporal. A análise da hemodinâmica consistiu da pressão ventricular sistólica esquerda (LVSP) e a pressão do final da diástole ventricular esquerda (LVEDP), assim como suas derivadas temporais (± dP/dt). A avaliação do estresse oxidativo foi realizada através da lipoperoxidação (medidas de quimiluminescência -QL- e substâncias reativas ao ácido tiobarbitúrico – TBARS) e oxidação das proteínas (carbonilas). O metabolismo da glutationa também foi avaliado (GSH/GSSG), juntamente com a capacidade antioxidante total (TRAP). A atividade das enzimas antioxidantes (superóxido dismutase - SOD, catalase -CAT, glutationa peroxidase - GPx, glutationa redutase – GR, e glutationa –S- transferase – GST)

também foram medidas. O metabolismo do óxido nítrico (NO), avaliado através da medida dos nitritos e nitratos, e a concentração tecidual de peróxido de hidrogênio (H_2O_2) também foram quantificadas. As medidas de expressão de proteínas foi realizada pelo método de Western blot, tendo como alvo as seguintes proteínas: Cu/Zn SOD, catalase, GST, receptor do fator de crescimento "*insulin like"*-I (IGF-IR), Akt e fosfo-Akt, GSK-3β e fosfo-GSK-3β, c-Jun e c-Fos. O tramento com a L–tiroxina foi efetivo no desenvolvimento do hipertireoidismo. O grupo tratado com a L–tiroxina demonstrou maiores valores de massa cardíaca, LVSP e LVEDP, com regressão destes parâmetros no grupo hipertireoideo+vitamina E. A lipoperoxidação, a oxidação das proteínas, a depleção de GSH e o consumo dos antioxidantes totais (TRAP) aumentaram no grupo hipertireoideo. O tratamento com a vitamina E preveniu estas alterações. A atividade e a expressão das enzimas antioxidantes se elevaram com o hipertireoidismo, exceto a catalase. No entanto, a administração da vitamina E reduziu a atividade destas enzimas, exceto da GST que apresentou atividade e expressão mais elevadas. Os nitritos e nitratos e as concentrações de H_2O_2 foram maiores no grupo hipertireoideo em relação ao grupo controle. Estes valores regrediram no grupo hipertireoideo+vitamina E. As expressões de proteínas do IGF-IR, fosfo-Akt, fosfo-GSK-3β, c-Jun e c-Fos foram maiores no grupo hipertireoideo, porém diminuídos pela ação da vitamina E. Estes dados sugerem não apenas a relação do hipertireoidismo com o estresse oxidativo, mas também uma ação dos radicais livres na ativação de vias intracelulares de sinalização, mediando processos de crescimento celular, como a hipertrofia cardíaca induzida pela tiroxina.

1. INTRODUÇÃO

1.1. Hipertireoidismo

Os hormônios da tireóide regulam o metabolismo basal dos tecidos, tais como o músculo esquelético e cardíaco, fígado, rim e cérebro (Venditti & Di Meo, 2006). A elevação dos hormônios da tireóide é denominada de hipertireoidismo. Os sinais e sintomas do hipertireoidismo freqüentemente ocorrem quando os tecidos são expostos a quantidades excessivas de hormônios tireoidianos. Os sinais clássicos incluem intolerância ao calor, taquicardia, perda de peso, fraqueza, labilidade emocional e tremores (Klein & Ojamaa, 2001). As causas mais comuns de hipertireoidismo são as doenças auto-imunes como a doença de Graves, a qual é decorrente de anticorpos circulantes contra os receptores de TSH. Tipicamente, há um bócio difuso que hipersecreta triiodotironina (T_3) e tiroxina (T_4). O defeito subjacente parece ser uma disfunção nos linfócitos T supressores que permitem a produção de um anticorpo contra esse receptor (Robbins *et al.*, 2005). Outros distúrbios que levam ao hipertireoidismo incluem o bócio tóxico multinodular, o adenoma tóxico, o carcinoma de tireóide e, raramente, um adenoma hipofisário secretor de TSH (hipertireoidismo secundário) (Henry,1999). Os efeitos cardíacos estão entre as mais constantes manifestações que os pacientes hipertireoideos apresentam, destacando-se o aumento do débito cardíaco, causado pela maior contratilidade cardíaca devido ao maior consumo de oxigênio, taquicardia, cardiomegalia e arritmia. O hipertireoidismo também pode causar a disfunção diastólica seguida de uma insuficiência cardíaca, chamada de cardiomiopatia tireotóxica dilatada (Robbins

et al., 2005). Além disso, Asahi *et.al,* em 2001 demonstraram a participação do sistema renina-angiotensina na disfunção cardíaca desenvolvida no hipertireoidismo.

1.2. Hipertireoidismo e Estresse Oxidativo

A aceleração do metabolismo basal e energético representa uma das mais importantes ações dos hormônios da tireóide (Venditti & Di Meo, 2006). Alguns autores sugerem que o hipertireoidismo está associado com a produção de radicais livres e com o aumento dos níveis de lipoperoxidação (Gredilla *et. al*, 2001). Da mesma forma, o estado hipometabólico do hipotireoidismo leva a uma diminuição na produção das EAO e de seus conseqüentes danos às proteínas, aos lipídios e ao DNA (Lopez-Torres *et al*., 2000). A capacidade total de peroxidação da mitocôndria é influenciada pelo estado tireoidiano do corpo. As variações dos níveis hormonais tireoidianos modulam o estresse oxidativo celular *in vivo*, principalmente, devido a sua ação sobre a mitocôndria (Guerrero *et al*., 1999). Desta maneira, a influência sobre a função mitocondrial, estimulando o consumo de oxigênio e a fosforilação oxidativa, torna essa organela um dos principais locais de produção das EAO, por ação dos hormônios tireoidianos (Sawyer et al., 2002). Quando a L-tiroxina é administrada aos animais, as mitocôndrias, de quase todas as células do organismo, aumentam em tamanho e número. Além disso, a superfície total da membrana interna mitocondrial, a atividade das enzimas oxidativas e os elementos do sistema de transporte de elétrons também aumentam proporcionalmente ao aumento do metabolismo basal. Uma das enzimas que tem sua atividade

elevada, em resposta aos hormônios da tireóide, é a Na^+, K^+–ATPase (bomba Na^+-K^+). Esta, por sua vez, intensifica o transporte de íons sódio e potássio através das membranas celulares, consumindo energia e aumentando a quantidade de calor produzida no organismo. Este processo poderia constituir um dos mecanismos pelos quais os hormônios da tireóide estimulam o metabolismo basal (Guyton & Hall, 2002).

O consumo de oxigênio e a produção de radicais livres ocorrem, preferencialmente, nas membranas lipídicas da mitocôndria. Desta forma, a sensibilidade ao dano oxidativo das membranas celulares depende muito da concentração de ácidos graxos insaturados, sendo os poliinsaturados os mais suscetíveis ao ataque das EAO. A mudança na composição lipídica das membranas celulares está intimamente correlacionada com as concentrações plasmáticas dos hormônios tireoidianos (Gredilla *et al*., 2001). Guerreiro *et al*., em 1999, demonstraram diminuição na concentração de ácido graxo insaturado nas frações lipídicas de animais hipotireoideos, desfavorecendo o ataque das EAO sobre as membranas celulares.

A razão glutationa reduzida (GSH)/glutationa oxidada (GSSG) está diminuída nos hipertireoideos, da mesma forma que a glutationa reduzida mitocondrial. Essa situação é revertida pela administração de propiltiouracil, usado no tratamento do hipertireoidismo, que aumenta a razão GSH/GSSG e restaura os valores da glutationa reduzida mitocondrial aos níveis normais. Este fato confirma o papel importante dos hormônios da tireóide na regulação do estresse oxidativo mitocondrial (Das & Chainy, 2004).

As enzimas antioxidantes superóxido dismutase (SOD) e glutationa peroxidase (GPx) têm suas atividades aumentadas no fígado de ratos

hipertireoideos, sugerindo uma resposta ao dano oxidativo (Venditti & De Meo *et al*., 2006).

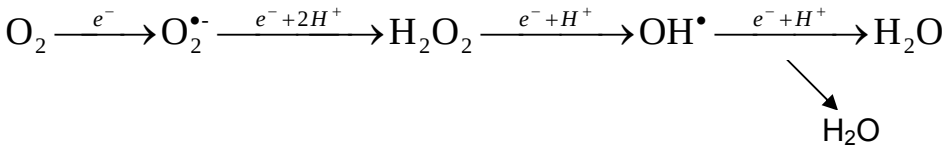
Em suma, o estresse oxidativo pode ser modulado de acordo com as concentrações séricas dos hormônios tireoidianos, e o hipertireoidismo pode aumentar a LPO e influenciar nas concentrações e atividade das moléculas antioxidantes (Niwa *et al.*, 2003).

1.3. Espécies Ativas de Oxigênio (EAO), Sistemas de Defesa Antioxidante e Estresse Oxidativo

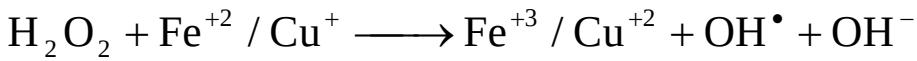
A geração das espécies ativas de oxigênio (EAO) ocorre, principalmente, durante os processos de oxidação biológica, dentre os quais, podemos destacar a respiração celular acoplada à fosforilação oxidativa, para formação de ATP na mitocôndria. O oxigênio (O_2) é reduzido até água (H_2O), recebendo quatro elétrons de uma só vez pela citocromo oxidase. Entretanto, em razão de sua configuração eletrônica, o oxigênio tem uma forte tendência a receber um elétron de cada vez gerando compostos intermediários muito reativos, como visto na reação 1A. Destes compostos intermediários, pode-se destacar o ânion radical superóxido ($O_2^{\bullet -}$), o peróxido de hidrogênio (H_2O_2) e o radical hidroxil ($OH^{\bullet}$). A formação destas moléculas ocorre em aproximadamente 5% de todo o processo de redução do oxigênio até água. A reação do peróxido de hidrogênio com íon ferroso ou cúprico é chamada de reação de Fenton, a qual leva à produção do radical hidroxil, muito reativo (reação 1B). O radical hidroxil também pode ser formado a partir da reação do ânion superóxido com o peróxido de hidrogênio em presença de íons divalentes de metais de transição, reação esta descrita por

Haber-Weiss (reação 1C). As EAO são fortemente oxidantes e podem apresentar pelo menos um elétron desemparelhado, quando isso ocorre são denominadas de radicais livres. Os radicais livres são definidos como qualquer espécie química capaz de existir independentemente que contenha um ou mais elétrons desemparelhados (Halliwell & Gutteridge, 1999). Ainda, deve ser relatado o estado excitado do oxigênio denominado de oxigênio "singlet", que também pode causar danos à célula (Halliwell, 2000).

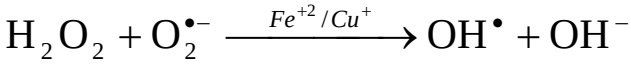
(A)



(B)



(C)



Reação 1: (A) Formação das EAO, a partir da redução do O_2. (B) Reação de Fenton. (C) Reação de Haber – Weiss

A magnitude da reatividade das EAO está relacionada com seus elétrons desemparelhados que lhes conferem a instabilidade característica. As reações com os radicais são muito rápidas e ocorrem em cadeia, já que os elétrons desemparelhados favorecem as colisões moleculares. O potencial reativo das

EAO é bem ilustrado na lipoperoxidação (LPO). A LPO é um processo de reação das EAO com os lipídios presentes nas membranas celulares que envolve não somente a renovação das membranas celulares, como também a biossíntese das prostaglandinas e leucotrienos (Halliwell, 2000). As proteínas também sofrem reações oxidativas iniciadas pelas EAO, que levam a alterações nas suas estruturas. Processos como fragmentação, agregação, maior susceptibilidade a proteases, exemplificam algumas das ações dos radicais livres sobre as proteínas (YU, 1994). Pode haver também um ataque ao DNA nuclear, sobretudo, pelo radical hidroxil, formando a 8-hidroxideoxiguanosina. O DNA mitocondrial também é bastante atingido, uma vez que a mitocôndria é a grande fonte de EAO (YU, 1994).

Além das EAO, há outros radicais livres que desempenham um importante papel na fisiologia e patologia humana, são as espécies ativas do nitrogênio (EAN). O óxido nítrico (NO) é radical livre, uma vez que possui um elétron desemparelhado. Esta característica é limitada, pois não são conhecidas reações de propagação do NO. Por outro lado, o NO reage facilmente com o radical superóxido produzindo o peroxinitrito ($ONOO^-$) (El-Helou *et al*., 2005). O NO é sintetizado nos organismos vivos, principalmente por um grupo de enzimas denominado óxido nítrico sintases (NOS) que convertem o aminoácido L-arginina em L-citrulina e NO. A atividade da NOS é controlada por citocinas, efetores e fatores de sinalização do NO para conexão do sistema imunológico com os sistemas cardiovascular, nervoso e endócrino (Bergendi *et al*., 1999). O NO é um gás hidrofóbico e, uma vez gerado, é capaz de difundir-se através da membrana plasmática das células endoteliais. O NO é responsável pelo controle do tônus muscular, onde atua como Fator Relaxante Derivado do Endotélio

(EDRF), produzindo vasodilatação e inibindo alguns processos como a agregação plaquetária, a adesão de leucócitos ao endotélio e a produção de endotelina (peptídio com potente ação vasoconstritora). O NO é liberado quando ocorre aumento do fluxo sangüíneo, do estresse de cisalhamento (*shear stress*), ou da pressão do sangue sobre o vaso, provocando o relaxamento do mesmo. Alguns dos efeitos fisiológicos do NO podem ser mediados pela formação do intermediário S-nitroso-glutationa que é um conjugado de glutationa e NO (Cristofanon *et al.*, 2006).

Os organismos aeróbios possuem sistemas de defesa para se protegerem contra os efeitos causados pelas EAO. Substâncias que neutralizam a ação dos radicais livres na LPO, levando à formação de produtos menos tóxicos, são chamadas de seqüestradoras (*scavengers*), como as enzimas antioxidantes e os tocoferóis. Aquelas que absorvem a energia de excitação dos radicais livres são chamadas de *quenchers,* tais como os carotenóides e o ácido ascórbico (Yu, 1994; Singal *et al*., 2000).

O sistema de defesa antioxidante é composto de elementos não-enzimáticos e enzimáticos. Dentre os compostos não-enzimáticos destaca-se a vitamina E ou α-tocoferol, o qual tem importância pelo seu efeito antioxidante e é comumente consumido na dieta. Há a forma γ-tocoferol que pode também apresentar uma capacidade antioxidante (Figura 1) (Devaraj *et al.,* 2005). A eficiência destas moléculas reside no fato de apresentarem a capacidade de inibir a propagação das reações desencadeadas pela LPO, o que é facilitado por sua localização nas membranas. Dados epidemiológicos destacam que o uso da vitamina E pode prevenir algumas doenças cardiovasculares, como a

aterosclerose, principalmente pela inibição da oxidação de lipoproteínas como o LDL (lipoproteína de baixa densidade) (Pryor, 2000). A vitamina C (ácido ascórbico), um potente antioxidante, desempenha um papel muito importante nos processos de regeneração do α-tocoferol (Reação 2). O β-caroteno, precursor da vitamina A, e o tripeptídio glutationa, presente em grandes concentrações na maioria das células eucariontes, somam-se aos demais antioxidantes não-enzimáticos (Cristofanon *et al.*, 2006).

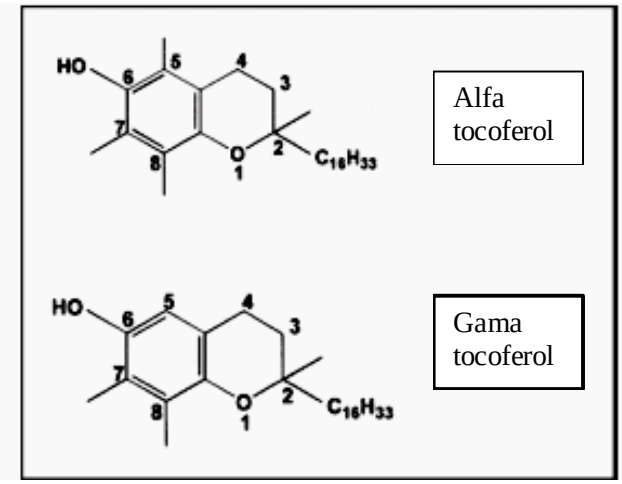
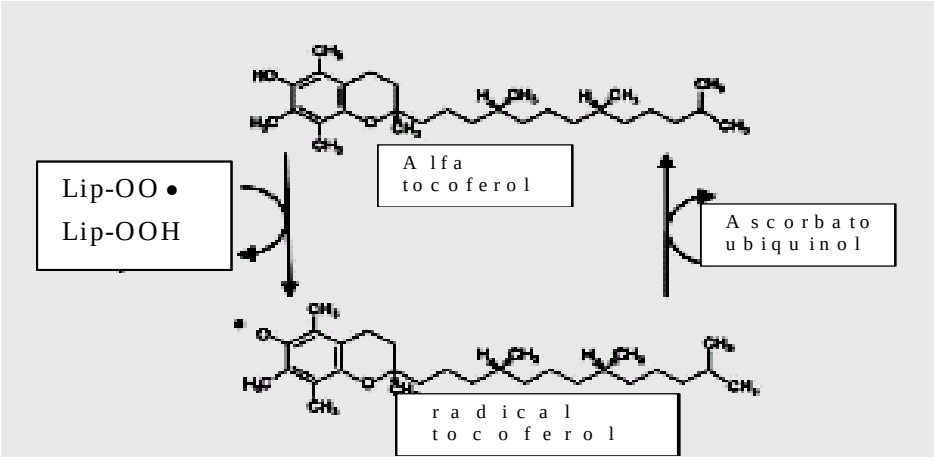


Figura 1: Estrutura química do α-tocoferol e Ɣ-tocoferol (Devaraj *et al.,* 2005)



Reação 2: Regeneração do α-tocoferol por ação da vitamina C.

Em sinergismo com a ação antioxidante dos compostos aludidos acima, estão as enzimas antioxidantes. A superóxido dismutase (SOD) é a enzima que catalisa a reação entre dois íons superóxido (dismutação) para formar peróxido de oxigênio (Reação 3A). Esta reação pode ocorrer espontaneamente em pH fisiológico, porém, na presença da SOD, a velocidade desta reação é 10^4 vezes maior (Yu, 1994; Halliwell & Gutteridge, 1999). A catalase (CAT) e a glutationa peroxidase (GPx) são enzimas que catalisam a redução dos peróxidos (peróxido de hidrogênio e de hidroperóxidos orgânicos), impedindo assim a formação de radical hidroxil e o conseqüente dano celular (Reações 3B e 3C). A glutationa redutase recicla o dissufeto de glutationa (GSSG) em glutationa reduzida (GSH) (Reação 3D) (Tsutsui *et al*., 2001). As glutationa-S-transferases (GSTs) são enzimas que agem na detoxificação de agentes eletrofílicos, como os radicais livres, e xenobióticos (exemplo cloro dinitro benzeno) (Reação 3E) (Habig, 1974).

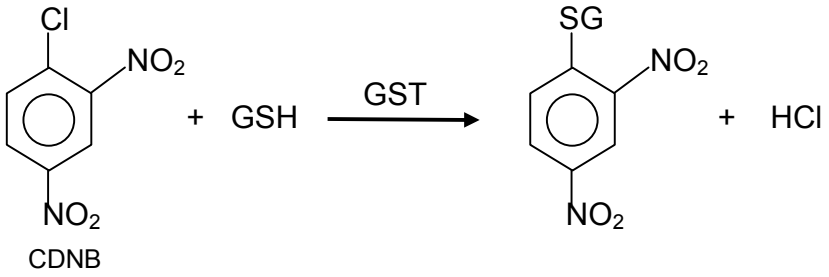
(A) $2H^+ + 2O_2^{\bullet -} \xrightarrow{SOD} O_2 + H_2O_2$

(B) $2H_2O_2 \xrightarrow{Catalase} O_2 + H_2O$

(C) $R - OOH + 2GSH \xrightarrow{GPx} R - OH + GSSG + H_2O$

(D) $GSSG + 2NADPH \xrightarrow{GR} 2GSH + 2NADP^+$

(E)



Reação 3: (A) superóxido dismutase (SOD), (B) catalase, (C) glutatina peroxidase (GPx), (D) regeneração da glutationa reduzida pela glutationa redutase (GR), (E) detoxificação do cloro dinitrobenzeno (CDNB) através da glutationa S-transferase (GST).

O estresse oxidativo pode ser definido como “um distúrbio do equilíbrio pró-oxidante/antioxidante em favor dos pró-oxidantes, levando ao dano potencial” (Sies, 1985). Em princípio, o estresse oxidativo pode resultar da diminuição dos antioxidantes, tais como das enzimas de defesa antioxidante ou da deficiência nutricional de antioxidantes (α-tocoferol, ácido ascórbico, aminoácidos contendo o enxofre necessário para a síntese de glutationa, ou riboflavina necessária para a produção de flavina adenina dinucleotídeo (FAD), um cofator da glutationa redutase). A produção aumentada de EAO e EAN também pode ser causa do estresse oxidativo, levando ao dano de biomoléculas tais como o DNA, as proteínas e os lipídios (Halliwell & Gutteridge, 1999, Singal *et al*, 2000).

1.4. EAO no processo de sinalização celular

Estudos recentes demonstram o papel importante das EAO nos eventos de sinalização intracelular. Há evidências de geração de EAO induzida por ligantes, além de rotas de sinalização ativadas por radicais livres (Dröge, 2002). Existem modelos que explicam os mecanismos de transdução de sinal pelos radicais livres baseados nas alterações do estado redox celular e na oxidação de proteínas (Liu *et al.*, 2005).

Há dois principais mecanismos de ação que podem explicar, de modo geral, a sinalização intracelular mediada pelas EAO. O primeiro está baseado nas alterações intracelulares do estado redox. O citosol normalmente é mantido em condições fortemente reduzidas, através dos grupos tióis que são

representados, principalmente, pela GSH. As alterações nas concentrações de GSH parecem regular a sinalização redox, e tais modificações nos seus níveis podem ser bem representadas pela razão da forma reduzida da glutationa (GSH) pela sua forma oxidada (GSSG) (Cristofanon *et al.*, 2006). O segundo mecanismo está associado às alterações conformacionais e funcionais das proteínas, causadas pela ação oxidativa dos radicais livres. As modificações nas estruturas das proteínas ocorrem preferencialmente em resíduos de aminoácidos que possuem grupos tiós e derivados, tal como a cisteína. Estas alterações na conformação nativa das proteínas induzem a dimerização e a interação com outros domínios protéicos ou com complexos metálicos distintos. Isto pode acarretar a modulação das funções de fatores de transcrição ou da atividade enzimática (Dröge, 2002; Liu *et al.,* 2005).

As citocinas e os fatores de crescimento podem ativar a produção de EAO ao se ligarem nos seus respectivos receptores. Após a ligação ao seu receptor, o fator de necrose tumoral-α (TNF-α) estimula a produção de radicais livres na mitocôndria, os quais ativam o fator de necrose *kappa beta* (NFκB). Esta sinalização parece estar envolvida no controle da apoptose celular (Dröge, 2002). O radical livre que se destaca neste mecanismo é o ânion radical superóxido ($O_2^{\bullet -}$), o qual induz a apoptose. Esta ação é inibida pelo aumento da expressão gênica da manganês-superóxido dismutase (MnSOD), enzima responsável pela eliminação desta EAO. Este mecanismo de apoptose parece estar também vinculado à depleção dos níveis de glutationa (GSH) (Liu *et al.*, 2005).

Alguns fatores de crescimento induzem a geração de EAO pela ativação de seus receptores tirosina quinase (RTK). Destacam-se o fator de crescimento derivado das plaquetas (PDGF), o fator de crescimento "*insulin like*-I" (IGF-I) e o fator de crescimento epidermal (EGF) (Dröge, 2002). A angiotensina II também estimula a produção de EAO, através da ativação da NADPH oxidase. As EAO podem desencadear a ativação dos membros da família das proteínas quinases ativadas por mitógeno (MAPK) (Figura 2) e induzir o desenvolvimento da hipertrofia ou da apoptose das células cardíacas, de acordo com as concentrações destas espécies no meio intracelular (Giordano, 2005).

A Akt (proteina quinase B) é uma via de fosforilação que pode ser rapidamente ativada, quando as células são expostas ao estresse oxidativo, principalmente pela ação do H_2O_2 (Cai *et al*., 2003; Yang *et al*., 2006). A Akt pertence à família das proteínas quinases serina/treonina que são classicamente estimuladas pelo receptor tirosina quinase, mediada pela ação da fosfatidilinositol 3-quinase (PI3K). A Akt é uma via de sinalização intracelular que pode estar relacionada com o crescimento da massa cardíaca, podendo agir através da supressão da via de apoptose dependente da glicogênio sintase quinase-3β (GSK 3β), promovendo o estabelecimento da hipertrofia cardíaca (Antos *et al.*, 2002).

As quinases ERK (quinase regulada por sinal extracelular), p38 e JNK (c-jun-NH2-terminal quinase) podem ser ativadas por substâncias oxidantes como peróxido de hidrogênio e radical superóxido, e desencadear o crescimento celular. O peróxido de hidrogênio também está envolvido na estimulação da ativação da proteína ativadora (AP 1), que é um heterodímero formado a partir dos fatores de transcrição c-Jun e c-Fos (Liu *et al.*, 2005). See e colaboradores

(2004) sugerem que a inibição da MAPK p38 pode ser uma perspectiva de terapia no tratamento da disfunção do cardiomiócito observada na insuficiência cardíaca, já que estas vias de sinalização intracelular estão envolvidas com a hipertrofia (Liu *et al*., 2005).

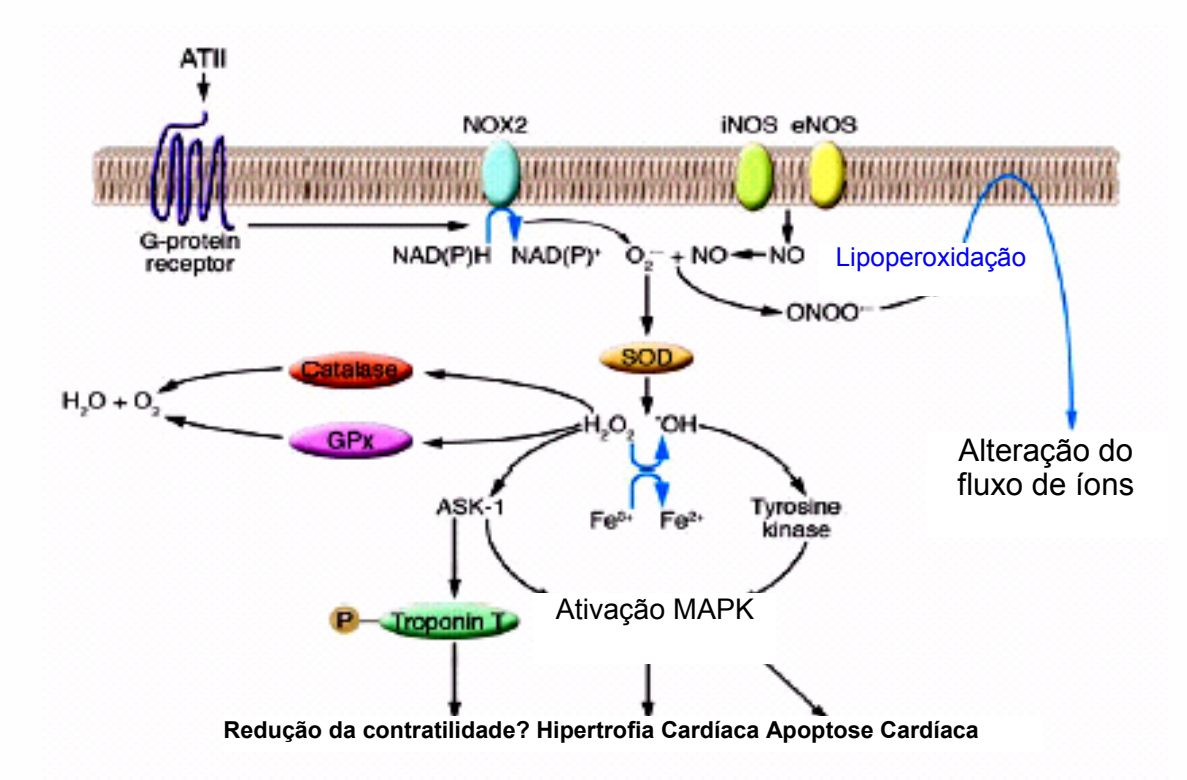


Figura 2: Esquema proposto para o desenvolvimento de hipertrofia cardíaca induzida pela ativação da MAPK (Giordano, 2005).

1.5. Hipertrofia Cardíaca, Sinalização Intracelular e Hipertireoidismo

O desenvolvimento da hipertrofia é um dos principais mecanismos pelos quais o coração busca compensar sobrecargas de volume ou de pressão. O objetivo dessa compensação é manter o estresse mecânico sistólico sobre a parede do ventrículo esquerdo (pré-carga) dentro da normalidade (Berenji *et al.*, 2005), mantendo uma força contrátil suficiente para vencer essa carga. Inicialmente, a hipertrofia resulta em mais miofibrilas disponíveis para contração, e também em aumento do número de mitocôndrias que suprem o cardiomiócito de ATP adicional (Brady & Terzic, 2000). Com isso, ocorre aumento da contratilidade no coração hipertrófico. Entretanto, persistindo a sobrecarga ao longo do tempo, ocorre deterioração da função contrátil, dilatação do coração e desenvolvimento de insuficiência cardíaca (Wakatsuki *et al*., 2004). Essas alterações têm como substrato morfológico uma série de modificações estruturais que ocorrem no coração hipertrófico: padrões anormais da banda-Z dos sarcômeros, múltiplos discos intercalares, núcleos aumentados (poliplóides), abundância de ribossomos e numerosas mitocôndrias (Hu *et al*., 2005). A hipertrofia envolve aumento no comprimento e espessura dos cardiomiócitos, expressão gênica alterada e modificações na composição das proteínas contráteis (Brady & Terzic, 2000).

As bases celulares e moleculares do aumento da massa cardíaca envolvem a hipertrofia dos cardiomiócitos existentes e vias de sinalização intracelular que desencadeiam este crescimento do tecido cardíaco (Heineke &

Molkentin, 2006). Existem vários possíveis mediadores dos processos pelos quais as sobrecargas de volume ou de pressão estimulam o crescimento celular. Dentre eles, estão os receptores de estiramento; os fatores de crescimento dos fibroblastos (FGF), o fator de crescimento "*insulin like*"-I (IGF-I); neurotransmissores e hormônios circulantes, como os agonistas α e β-adrenérgicos, a angiotensina II, a tiroxina, e a insulina; segundos mensageiros intracelulares, como AMP cíclico, diacilglicerol, inositol trifosfato e cálcio; proto-oncogenenes *c-fos*, *c-myc* e *c-jun* (Frolich *et al.*, 1992). Esses fatores de crescimento podem atuar ativando proteínas de transdução intracelular e fatores de transcrição (Figura 3) (Heineke & Molkentin, 2006).

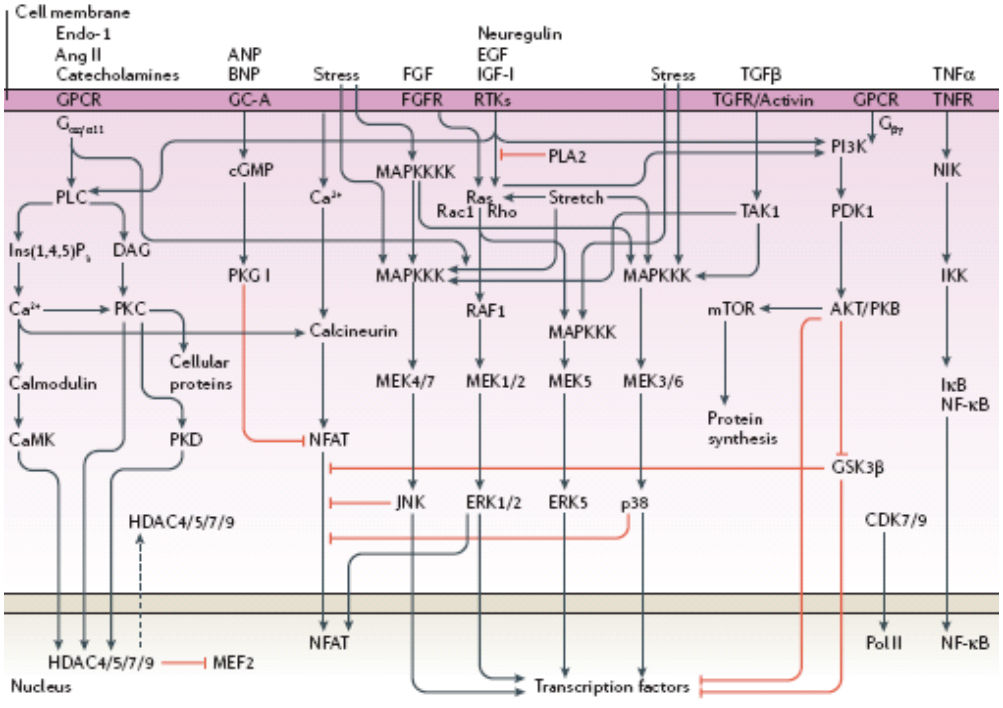


Figura 3: Diferentes vias de sinalização intracelular envolvidas no desenvolvimento da hipertrofia cardíaca (Heineke & Molkentin, 2006).

A modulação da expressão do IGF-I e de seus receptores nos cardiomiócitos é um dos eventos moleculares envolvidos na hipertrofia cardíaca. Este aumento efetivo da massa muscular do coração pode ser devido à elevação da síntese "*de novo*" de proteína causada pela ação do IGF-I (Kuo *et al.,* 2005). Lin et al*.,* 1999 mostrou que a L-tiroxina pode ativar a ERK através de um mecanismo dependente de proteína G em células CV-1 e Hela, mas a ativação deste mecanismo não está bem estabelecida no tecido cardíaco. A via de sinalização da Akt tem sido implicada na regulação do crescimento e da função cardíaca (Wilkins *et al*., 2004; DeBosch *et al*., 2006). A superexpressão cardíaca da Akt constitutiva resultou em hipertrofia concêntrica e aumento da contratilidade cardíaca (Condorelli *et al*., 2002). Dados recentes demonstraram a ativação da Akt via tiroxina no coração (Kuzman *et al*., 2005). A GSK-3β também apresenta um papel importante na regulação negativa da hipertrofia cardíaca. Camundongos que superexpressam GSK-3β, sujeitos à sobrecarga de pressão, demonstraram redução da hipertrofia cardíaca (Antos *et al*., 2002). No entanto, os mecanismos pelos quais estas vias são ativadas no hipertireoidismo ainda foram pouco explorados.

2. HIPÓTESE

O hipertireoidismo induz a hipertrofia cardíaca pelo seu estímulo sobre as vias de sinalização intracelular e os fatores de crescimento, usando como moléculas mediadoras para este processo as EAO.

3 .OBJETIVOS

Objetivo Geral

Avaliar o estresse oxidativo cardíaco, através de medidas de dano oxidativo e de defesa antioxidante enzimáticas e não-enzimáticas, correlacionando-o com a avaliação hemodinâmica e morfométrica, com a expressão de fatores de crescimento teciduais e com vias de sinalização sensíveis ao estresse oxidativo, em ratos submetidos ao hipertireoidismo crônico, assim como sob tratamento com antioxidante.

Objetivos Específicos

⇒ Induzir experimentalmente o hipertireoidismo através da administração da L-tiroxina no período de uma a quatro semanas, verificando a efetividade do tratamento pela dosagem das concentrações plasmáticas de hormônios tireoidianos e pela avaliação dos parâmetros morfométricos e hemodinâmicos.

⇒ Realizar medidas de avaliação do estresse oxidativo no tecido cardíaco dos distintos grupos experimentais: quimiluminescência, substâncias reativas ao ácido tiobarbitúrico, carbonilas, capacidade antioxidante total, metabolismo da glutationa, atividade e concentração das enzimas antioxidantes, peróxido de hidrogênio e nitritos e nitratos.

⇒ Avaliar a expressão o receptor de IGF-I, juntamente com fatores de transcrição, c-Jun, c-Fos, e com a cascata de sinalização da Akt e GSK-3β neste modelo experimental.

4. METODOLOGIA

4.1. Animais

Foram utilizados, no trabalho, ratos machos Wistar, com peso médio de 250 ± 20 gramas. Os animais provenientes do Biotério do Instituto de Ciências Básicas da Saúde (ICBS) da UFRGS, foram mantidos em caixas plásticas de 270 x 260 x 310 mm, com 4 ratos cada, com o assoalho recoberto com serragem. Receberam alimentação e água à vontade e foram mantidos sob períodos de 12 horas luz/12 horas escuro e sob temperatura de 22°C. Os procedimentos experimentais foram realizados de acordo com as normas do Colégio Brasileiro de experimentação animal.

4.2. Indução do Hipertireoidismo

O hipertireoidismo crônico foi obtido mediante administração de L-tiroxina (0,15 µmo/L) -12 mg T4 dissolvido em 50 mL de Tris para um volume final de um litro de água - *ad libitum* durante uma a quatro semanas, na água de beber oferecida aos ratos (Ladenson *et al.,* 1986).

4.3. Grupos e Protocolos Experimentais

4.3.1**.** Artigo 1- Myocardial Antioxidant Enzyme Activities and Concentration and Glutathione Metabolism in Experimental Hyperthyroidism *(Molecular and Cellular Endocrinology 249 (2006) 133-139)*

Foram utilizados dois grupos experimentais (n=10):

1) **Hipertireoideo**: animais que foram submetidos à ingestão de L-tiroxina na água de beber, no período de uma a quatro semanas.

2) **Controle**: animais que receberam apenas o veículo da L-tiroxina (Tris) na água de beber, no período de uma a quatro semanas.

4.3.2. Artigo 2: Oxidative stress activates insulin-like growth factor I receptor protein expression mediating cardiac hypertrophy induced by thyroxine (*submetido em 2006 para Molecular and Cellular Biochemistry, aguardando o processo de revisão*)

Foram utilizados quatro grupos experimentais (n=10):

1) **Hipertireoideo**: animais que foram submetidos à ingestão de L-tiroxina na água de beber, no período de quatro semanas.

2) **Controle**: animais que receberam apenas o veículo da L-tiroxina (Tris) na água de beber e óleo mineral por via subcutânea, no período de quatro semanas.

3) **Vitamina E**: animais que receberam vitamina E (diluída em óleo mineral), administrada por via subcutânea, no período de quatro semanas.

4) **Hipertireoideo+Vitamina E**: animais que foram submetidos à ingestão de L-tiroxina, uma a quatro semanas. Tratamento este, concomitante ao tratamento com vitamina E administrada por via subcutânea, no período de quatro semanas.

4.3.3. Artigo 3: Oxidative stress role in the AKT pathway activation in experimental hyperthyroidism (*submetido em 2006 para Journal Molecular Cardiology, aguardando o processo de revisão*)

Foram utilizados quatro grupos experimentais (n=10):

1) **Hipertireoideo**: animais que foram submetidos à ingestão de L-tiroxina na água de beber no período de quatro semanas.

2) **Controle**: animais que receberam apenas o veículo da L-tiroxina (Tris) na água de beber e óleo mineral por via subcutânea, no período de quatro semanas.

3) **Vitamina E**: animais que receberam vitamina E (diluída em óleo mineral), administrada por via subcutânea, no período de quatro semanas.

4) **Hipertireoideo+Vitamina E**: animais que foram submetidos à ingestão de L-tiroxina, uma a quatro semanas. Tratamento este, concomitante ao tratamento com vitamina E administrada por via subcutânea, no período de quatro semanas.

4.4. Protocolo Experimental

O hipertireoidismo foi induzido através da administração de L-tiroxina (T_4) durante o período de uma a quatro semanas. Foi coletado sangue por punção

cardíaca, no término de cada referido tempo de tratamento, para análise da tiroxina plasmática. No seguimento do protocolo experimental, os animais foram decapitados, e tiveram removidos seus corações para avaliar o índice de hipertrofia cardíaca. Os corações, então, foram homogeneizados para avaliar o estresse oxidativo e a expressão das enzimas antioxidantes, do receptor de IGF, Akt, GSK 3β, c-Jun e c-Fos.

O grupo hipertireoideo+vitamina E foi tratado com vitamina E, administrada por via subcutânea (15 mg/kg/dia em óleo mineral), concomitante ao tratamento com L-tiroxina. Foram avaliados, neste grupo, os mesmos parâmetros supracitados.

4.5. Dosagem Hormonal

Nos animais anestesiados (quetamina 90mg/kg; xilazina 10mg/kg, i.p.), foi realizada a coleta de 1,5 mL de sangue por punção cardíaca. O sangue coletado foi centrifugado por 10 minutos a 1000 g em centrífuga refrigerada (Sorvall RC 5B – Rotor SM 24). O plasma dos animais foi separado e congelado em freezer a -80ºC para posteriores dosagens dos hormônios tireoidianos. O método utilizado para dosar o hormônio T4 foi o de quimiluminescência por imunoensaio competitivo. O aparelho utilizado para esta medida foi o IMMULITE 2000. Os resultados foram expressos em ng/mL. As dosagens hormonais foram realizadas pelo Laboratório Weinmann.

4.6. Avaliações Hemodinâmicas e Cálculo do Índice de Hipertrofia Cardíaca

Após o tratamento, os animais foram pesados e anestesiados (quetamina 90 mg/kg; xilazina 10 mg/kg, i.p.). Foi utilizado, para a canulação do ventrículo esquerdo via artéria carótida direita, um catéter de polietileno (PE50) conectado a um transdutor de pressão (Strain-Gauge-Narco Biosystem Transducer RP-155, Houston, Texas, USA) e acoplado a um amplificador de sinais (Pressure Amplifier HP 8805C). O catéter foi inserido até o ventrículo e sua posição foi determinada pela observação da onda característica de pressão ventricular. Após 5 minutos de estabilização, foram registradas a pressão sistólica ventricular esquerda (PSVE) e a pressão diastólica final do ventrículo esquerdo (PDFVE). Os sinais analógicos da pressão foram digitalizados (CODAS - Data Acquisition System, PC 486) com taxa de amostragem de 1000 Hz. Este programa permite a derivação da onda de pressão ventricular esquerda e detecção dos valores máximos e mínimos destas curvas, batimento a batimento, fornecendo os valores das derivadas positivas (+dP/dt - índice de contratilidade) e negativas (-dP/dt - índice de relaxamento), expressas em mmHg/s (Li *et al*., 2000).

O índice de hipertrofia cardíaca oferece um indicativo do aumento da massa muscular do coração, o qual é uma característica importante a ser ressaltada no hipertireoidismo. Este foi calculado pela razão do peso em miligrama de tecido cardíaco por grama de peso corporal (Hu *et al.*, 2003).

4.7. Preparo dos Homogeneizados

Os corações foram rapidamente retirados e homogeneizados por 30 segundos em Ultra-Turrax, na presença de KCl 1,15% (5 mL/g de tecido) e de fluoreto de fenil metil sulfonil (PMSF), na concentração de 100 mmol/L em isopropanol (10 µL/mL de KCl adicionado). O PMSF é um inibidor de proteases e foi utilizado para que não ocorresse degradação das enzimas das quais a atividade foi medida. Em seguida, os homogeneizados foram centrifugados por 10 minutos a 1000 g em centrífuga refrigerada (Sorvall RC 5B-Rotor SM 24) e o sobrenadante foi retirado e congelado em freezer a -80ºC para as dosagens posteriores de LPO e atividade das enzimas antioxidantes (Llesuy *et al*., 1985). Para análise de Western blot, cerca de 100 mg do tecido cardíaco (ventrículos esquerdo e direito) foi rapidamente retirado e homogeneizado por 30 segundos em Ultra-Turrax, na presença do tampão de homogeneização (Tris-HCl 100mmol/L, EDTA 5 mmol/L, PMSF 1 mmol/L, Aprotinina 5 g/mL) (Laemmli *et al.,* 1970). Em seguida, os homogeneizados foram centrifugados por 20 minutos a 1000 g em centrífuga refrigerada (Sorvall RC 5B-Rotor SM 24) e o sobrenadante foi retirado e congelado em freezer a -80ºC.

4.8. Quantificação de Proteínas

As proteínas foram quantificadas pelo método descrito por Lowry e colaboradores, em 1951, que utiliza como padrão uma solução de albumina bovina na concentração de 1 mg/mL. A medida foi efetuada em espectrofotômetro a 625 nm e os resultados expressos em mg/mL.

4.9. Medidas de dano oxidativo

O dano oxidativo aos lipídios (lipoperoxidadação) foi avaliado através da quimiluminescência, iniciada pelo hidroperóxido de tert-butil (QL), e substâncias reativas ao acido tiobarbitúrico (TBARS). A QL foi medida em contador beta (LKB Rack Beta Liquid Scintilation Spectrometer). O meio de reação no qual foi realizado o ensaio consistiu em 3,5 mL de uma solução tampão de KCl 140mmol/L e fosfatos de sódio e potássio 20 mmol/L (pH 7,4). Os resultados foram expressos em contagens por segundo (cps), por miligrama de proteína, segundo Gonzalez-Flecha *et al*., (1991). A técnica de TBARS consiste na reação dos produtos da lipoperoxidação com o ácido tiobarbitúrico, para medir espectrofotometricamente a formação de um produto de coloração rósea. Utilizou-se como padrão o tetrametoxipropano. Os resultados foram expressos em nmoles de malondialdeído (MDA) por mg de proteína (Buege & Aust, 1978).

Para determinação das proteínas modificadas oxidativamente, utilizou-se o ensaio de determinação das carbonilas (Reznick & Packer, 1994). A técnica se baseia na reação das proteínas oxidadas do tecido com 2,4 dinitro fenil hidrazina (DNPH) em meio ácido, seguido de sucessivas lavagens com ácidos e solventes orgânicos e incubação final com guanidina. A absorbância das carbonilas foi medida em um espectrofotômetro da marca Varian, modelo Cary, a 360 nm. Os resultados foram expressos em nmoles por mg de proteína.

4.10. Determinação da Concentração da Glutationa Total e Reduzida

Na determinação da glutationa total, o tecido cardíaco foi desproteinizado com ácido perclórico 2 mol/L e centrifugado por 10 min a 1000 g. O sobrenadante foi neutralizado com hidróxido de potássio 2 mol/L. O meio de reação continha tampão fosfato 100 mmol/L (pH 7,2), ácido nicotinamida dinucleotídeo fosfato (NADPH) 2 mmol/L, glutationa redutase 0,2 U/mL, 5,5' ditiobis (2-nitro ácido benzóico) 70 µmol/L. Na determinação da glutationa reduzida, o sobrenadante neutralizado reagiu com 5,5' ditiobis (2-nitro ácido benzóico) 70 µmol/L e a leitura foi efetuada a 420 nm (Akerboom & Sies,1981).

4.11. Capacidade Antioxidante Total (TRAP)

O método consiste em incubar uma mistura de 2,2'-azo-bis (2-amidinopropano) (ABAP - 10 mmol/L), usado como uma fonte de radicais livres, com luminol (10 µmol/L) em tampão glicina (0,1 mmol/L - pH 8,6). Esta mistura foi levada ao contador e a luminescência surgiu da oxidação do luminol pelos radicais livres. A adição de Trolox (vitamina E hidrossolúvel) reduz quase que completamente a emissão de luz, produzindo um tempo de indução que está linearmente relacionado com a concentração de Trolox. Foi elaborada a curva de calibração com Trolox nas concentrações de 0,2 a 1 µmol/L. O homogeneizado de tecido foi adicionado ao invés do Trolox e se observou o tempo de indução que também está relacionado com a quantidade de homogeneizado adicionado. A capacidade total antioxidante foi avaliada medindo o tempo de indução de uma

dada quantidade de homogeneizado. A quantidade total de antioxidantes presente no tecido foi avaliada (em unidades de Trolox) pela interpolação da medida do tempo de indução com a curva de calibração obtida com o Trolox (Evelson *et al*., 2001).

4.12. Atividade das enzimas antioxidantes

Foram medidas as atividades das enzimas antioxidantes SOD, GPx, CAT e GST. A técnica utilizada neste trabalho para determinação da SOD está baseada na inibição da reação do radical superóxido com o pirogalol. O superóxido é gerado pela auto-oxidação do pirogalol em meio básico. A SOD presente na amostra em estudo compete pelo radical superóxido com o sistema de detecção. A oxidação do pirogalol foi detectada espectrofotometricamente a 420 nm. A atividade da SOD foi determinada medindo a velocidade de formação do pirogalol oxidado. No meio de reação foram adicionados tampão Tris 50 mmol/L (pH 8,2), pirogalol 24 mmol/L, catalase 30 μmol/L. Os resultados foram expressos em U SOD/mg proteína, segundo Marklund, 1985.

A enzima glutationa peroxidase (GPx) catalisa a reação de hidroperóxidos com a glutationa reduzida (GSH) para formar glutationa oxidada (GSSG) e o produto da redução do hidroperóxido. Logo, sua atividade pode ser determinada medindo-se o consumo de NADPH na reação de redução acoplada à reação da GPx. Mediu-se a atividade da glutationa peroxidase em espectrofotômetro a 340 nm, em um meio de reação que continha: solução tampão fosfato 140 mmol/L, EDTA 1 mmol/L (pH 7,5), NADPH 0,24 mmol/L; azida sódica 1 mmol/L, utilizada

para inibir a atividade da catalase; GSH 5 mmol/L; glutationa redutase (GR) 0,25 U/mL e, por fim, hidroperóxido de tert- butila 0,5 mmol/L (Flohé & Gunzler, 1984).

A atividade da catalase é diretamente proporcional à taxa de decomposição do peróxido de hidrogênio e obedece a uma cinética de pseudo-primeira ordem. Sendo assim, sua atividade pode ser medida através da avaliação do consumo do H_2O_2. Este teste consiste em avaliar a diminuição da absorbância no comprimento de onda de 240 nm, que é onde ocorre maior absorção pelo peróxido de hidrogênio. Para a realização deste ensaio foram utilizados: solução tampão-fosfato a 50 mmol/L (pH=7,4) e peróxido de hidrogênio 0,3 mol/L. Os resultados foram expressos em picomoles por miligramas de proteína (Aebi, 1984).

As glutationas transferases são um grupo de enzimas que catalisam reações de conjugação de glutationa com vários xenobióticos, tendo um importante papel na detoxificação de agentes alquilantes. Todas as transferases são ativas com o composto 1-cloro-2,4 dinitro benzeno (CDNB), sendo a conjugação deste com GSH utilizada para quantificar-se sua atividade. A formação do composto corado dinitro-fenil-glutationa (DNP-SG), foi medida espectrofotometricamente a 340 nm. Para realização deste ensaio foram utilizados solução tampão de fosfato de sódio 0,2 mol/L (pH = 6,5) e 150 μL de CDNB (20 mmol/L). A atividade da GST foi expressa em nmoles/min/mg de proteína (Mannervik & Gluthemberg, 1981).

4.13. Análise de nitritos e nitratos

Os níveis de nitritos e nitratos no tecido cardíaco foram medidos pela reação das amostras com o reagente de Griess. As amostras foram incubadas com cofatores enzimáticos (Tris 1 mol/L, pH 7,5; NADPH 0,02 mmol/L), glicose 6-fosfato (G6P) 5 mmol/L, glicose 6-P desidrogenase (G6PDH) 10 U/mL e nitrato redutase, (1,75 U/mL; Sigma, St. Louis, MO) por uma hora para conversão de nitrato em nitrito em temperatura ambiente. Os nitritos foram determinados pela reação das amostras com o reagente de Griess (1% sulfanilamina, 0.1% naftiletilenodiamina, 2,3 mL ácido ortofosfórico 85%). O total de nitrito tecidual (nitrito inicial mais o nitrito produzido a partir da redução do nitrato), foi estimado em 540 nm, a partir de uma curva padrão de nitrito de sódio (10^{-8} a 10^{-3} mol/L). Os resultados foram expressos em mmol/L (Granger *et al*., 1999).

4.14. Medida do peróxido de hidrogênio

O método está baseado na oxidação do vermelho de fenol pelo peróxido de hidrogênio mediada pela peroxidase de rabanete, resultando num produto que absorve em 610 nm. Fatias de tecido cardíaco foram incubadas por 30 minutos em tampão fosfato de potássio 10 mmol/L (NaCl 140 mmol/L e dextrose 5 mmol/L). O sobrenadante foi coletado e adicionado na solução tampão de vermelho de fenol 0,28 mmol/L e peroxidase de rabanete 8,5 U/mL. Após o intervalo de 5 minutos, foi adicionado NaOH 1 mol/L e efetuada a leitura a 610

nm. Os resultados foram expressos em nmoles de H_2O_2 por mg de proteína (Pick & Keisari, 1980).

4.15. Análise por "Western blot"

Neste método, quarenta microgramas de proteína foram expostos à eletroforese de gel monodimensional de dodecil sulfato de sódio-poliacrilamida (SDS-PAGE) num sistema descontínuo usando 12% (p/v) de gel separador e 5% (w/v) de gel fixador. As proteínas separadas foram transferidas através de eletroforese para membranas usando tampão Towbin modificado, contendo Tris 20 mmol/L, glicina 150 mmol/L, metanol 20% (v/v), SDS 0,02% (p/v) (pH= 8,3) numa unidade de transferência Bio-Rad resfriada (durante 1h em 220 mA). Após, os sítios de proteínas inespecíficas foram bloqueados através de 1h de incubação em solução bloqueadora (5% (p/v) de leite desnatado) em tampão Tris salina 0,1% (p/v), Tween-20. As membranas foram processadas por imunodetecção, usando-se os seguintes anticorpos primários: anticorpo policlonal anti-humano GST de cabra, anticorpo policlonal anti-Cu/Zn SOD (23 kDa) de coelho, anticorpo policlonal anti-CAT (65 kDa) de ovelha, anticorpo policlonal anti-IGF-IR (98 kDa) de coelho, anticorpo policlonal anti-Akt e fosfo-Akt de coelho (60 kDa), anticorpo policlonal anti-GSK 3β e fosfo- GSK 3β de coelho (47 kDa), anticorpo policlonal anti-c-Jun (39 kDa) e anticorpo policlonal anti-c-Fos (62 kDa). Como anticorpo secundário foi utilizado o anticorpo policlonal anti-humano, anticorpo policlonal anti-ovelha e anticorpo monoclonal anti-cabra (Santa Cruz Biotechnology, Santa Cruz, CA, USA). As membranas foram reveladas através do uso de substrato quimiluminescente, e as autorradiografias

geradas foram analisadas usando densitômetro de imagem (Imagemaster VDS CI, Amersham Biosciences Europe, IT). O resultado de cada membrana foi normalizado pelo método de Ponceau red (Klein *et al.,* 1995).

5. ANÁLISE ESTATÍSTICA

Com base nos resultados das análises, foram calculados as médias e os desvios padrões para cada uma das medidas realizadas e para cada um dos grupos estudados. Como os dados apresentaram distribuição normal, foi aplicada o teste t'studant e a análise de variância (ANOVA) de uma via para a comparação entre os grupos, complementado com o teste de Student-Newmann-Keuls quando necessário. A correlação entre a as variáveis foi analisada pela correlação de Pearson (*GraphPad Instat*, versão 3.0). As diferenças foram consideradas significativas quando a análise estatística apresentou $P < 0,05$. O cálculo do tamanho da amostra foi realizado através *Computer Programs for Epidemiologic Analyses* (PEPI versão 4.0).

6. RESULTADOS

Os resultados obtidos neste estudo foram descritos em forma de artigos e subdivididos de maneira que fossem submetidos a três revistas indexadas com fator de impacto acima de 1,0. Neste capítulo, tais resultados serão apresentados segundo o quadro abaixo**:**

Título do artigo	***Periódico***
"Myocardial Antioxidant Enzyme Activities and Concentration and Glutathione Metabolism in Experimental Hyperthyroidism"	*Molecular and Cellular Endocrinology 249: 133-139* ,2006.
"Oxidative stress activates insulin-like growth factor I receptor protein expression mediating cardiac hypertrophy induced by thyroxine"	*Submetido para: Molecular and Cellular Biochemistry*
"Oxidative stress role in the AKT pathway activation in experimental hyperthyroidism"	*A ser submetido para: Journal of Molecular and Cellular Cardiology*

6.1 Artigo 1: *Myocardial Antioxidant Enzyme Activities and Concentration and Glutathione in Experimental Hyperthyroidism (Molecular and Cellular Endocrinology 249: 133-139, 2006).*

ELSEVIER

Molecular and Cellular Endocrinology 249 (2006) 133–139

MCE
Molecular and Cellular Endocrinology

www.elsevier.com/locate/mce

Myocardial antioxidant enzyme activities and concentration and glutathione metabolism in experimental hyperthyroidism

A.S.R. Araujo[a], M.F.M. Ribeiro[a], A. Enzveiler[a], P. Schenkel[a], T.R.G. Fernandes[a], W.A. Partata[a], M.C. Irigoyen[a], S. Llesuy[b], A. Belló-Klein[a,*]

[a] *Departamento Fisiologia, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil*
[b] *Cátedra de Química General e Inorgánica, Facultad de Farmácia y Bioquímica, Universidad de Buenos Aires, Argentina*

Received 15 August 2005; received in revised form 9 February 2006; accepted 10 February 2006

Abstract

Hyperthyroidism was induced in rats by L-thyroxine administration (12 mg/L in drinking water, 4 weeks). Animals were assessed hemodynamically, and heart, lung, and liver morphometry were performed. Lipid peroxidation (LPO) and protein oxidation (carbonyls) were measured in heart homogenates. It was quantified glutathione (GSH) metabolism, and antioxidant enzyme activities its and protein expression (by Western blot). At the end of treatment, it was observed cardiac hypertrophy, elevation of left ventricular systolic and end diastolic pressures, lung and liver congestion. LPO and carbonyls were increased in the hyperthyroid group, and GSH was decreased by 46% in the fourth week. Myocardial oxidative stress time course analysis revealed that it was increased in the second week of treatment. Antioxidant enzyme activities elevation was accompanied by protein expression induction in the hyperthyroid group in the fourth week. These results imply that hyperthyroidism generates myocardial dysfunction associated with oxidative stress inducing antioxidant enzyme activities and protein expression.

Keywords: Hyperthyroidism; Lipid peroxidation; Antioxidant enzyme protein expression; Glutathione metabolism; Heart failure

1. Introduction

Reactive oxygen species (ROS), generated as by-products of oxidative metabolism in mitochondria, can interact with biomolecules and damage various cellular components. Aerobic organisms are endowed with antioxidant enzymes such as: superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), catalase (CAT). As non-enzymatic defense molecules we can highlight reduced glutathione (GSH), ascorbate, vitamin E and flavonoids (Hogg and Kalyanamaran, 1999). However, when ROS generation exceeds the antioxidant capacity of cells, oxidative stress develops, potentially causing tissue damage (Esterbauer et al., 1991; Venditti et al., 1996).

ROS have a high reactivity potential, therefore they are toxic and can lead to oxidative damage in cellular macromolecules such as proteins, lipids and DNA. Thus, ROS have been implicated in the pathophysiology of a large number of diseases. Evidence from experimental as well as clinical studies suggests the involvement of oxidative stress in the pathogenesis of cardiac dysfunction, such as heart failure (Singal and Iliskovic, 1998; Das and Chainy, 2001).

Thyroid hormones are the most important factors involved in the regulation of the basal metabolic state, as well as in the oxidative metabolism (Asayama and Kato, 1990). Changes in thyroxine (T_4) and triiodothyronine (T_3) levels are the main events of physiological modulation of the mitochondrial respiration process "in vivo". Thyroid hormones can cause many changes in the number and activity of mitochondrial respiratory chain components. This may result in increased generation of ROS (Mano et al., 1995; Guerrero et al., 1999).

Hyperthyroidism shows a hyperdynamic circulation, with increased cardiac output, increased heart rate, and decreased peripheral resistance. These cardiovascular manifestations of hyperthyroidism have been reproduced in rats under thyroid hormone treatment (Klein, 1990). The heart has a typically high aerobic metabolic rate, with large and plentiful mitochondria. However, the myocardial defense mechanism against oxygen toxicity is deficient and probably more susceptible to ROS attack.

* Corresponding author at: Sarmento Leite, 500, CEP: 90050-170, Porto Alegre, RS, Brazil. Tel.: +55 2151 33163621; fax: +55 2151 33163166.
E-mail address: belklein@ufrgs.br (A. Belló-Klein).

0303-7207/$ – see front matter © 2006 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.mce.2006.02.005

There is strong evidence in the literature, as mentioned above, that thyroid hormones lead to oxidative stress in the cardiac tissue. Nevertheless, there is scarce information about the modulation of protein expression of antioxidant enzymes, and their relationship to the glutathione metabolism in experimental hyperthyroidism. Thus, the purpose of the present study was to evaluate myocardial concentration of antioxidant enzymes Cu/Zn SOD, GST and CAT, glutathione metabolism changes, and oxidative damage in rats with heart failure induced by hyperthyroidism.

2. Material and methods

2.1. Animals

Twenty male Wistar rats (200 ± 20 g) were obtained from the Central Animal House at Universidade Federal do Rio Grande do Sul. The animals were housed in plastic cages (four animals each) and received water and pelleted food ad libitum. They were maintained under standard laboratory conditions (controlled temperature of 21 °C, 12 h light/dark cycle). Animals were weighed weekly to follow body weight gain. They were divided into two groups ($n = 10$): the control, which received water ad libitum, and hyperthyroid, which received thyroxine (T_4) (12 mg/L in drinking water) (Ladenson et al., 1986).

2.2. Hemodynamic measurements and cardiac hypertrophy

Cardiac hemodynamics was assessed from the first to fourth week of thyroxine treatment. In brief, the rats were anesthetized (ketamine 90 mg/kg; xylazine 10 mg/kg, i.p.) and the right carotid artery was cannulated with a PE 50 catheter connected to a strain gauge transducer (Narco Biosystem Pulse Transducer RP-155, Houston, TX, USA) linked to a pressure amplifier (HP 8805C, Hewlett Packard, USA). Pressure readings were taken in a microcomputer equipped with an analogue-to-digital conversion board (CODAS 1 kHz sampling frequency, Dataq Instruments Inc., Akron, OH, USA). The catheter was advanced into left ventricle (LV) for recording the left ventricular systolic pressure (LVSP, mmHg) and left ventricular end diastolic pressure (LVEDP, mmHg). The cardiac hypertrophy was evaluated by heart weight (in mg) to body weight (in g) ratio.

2.3. Tissue preparation

Four weeks after treatment, the rats were decapitated and the hearts were rapidly excised, weighed and homogenized (1.15%, w/v KCl and phenyl methyl sulphonyl fluoride (PMSF) 20 mmol/L) in Ultra-Turrax. The suspension was centrifuged at 600 × *g* for 10 min at 0–4 °C to remove the nuclei and cell debris (Llesuy et al., 1985) and supernatants were used for the assay of carbonyls, lipid peroxidation and enzymatic activity. At the moment of sacrifice, cardiac tissue samples were rapidly removed and frozen at −80 °C, for the evaluation of glutathione metabolism and enzyme expression. Lung and liver were also taken and weighed in order to estimate these organs congestion. These parameters were expressed as organs wet weight (in mg) to body weight (in g).

2.4. Thyroid hormone concentration

After sacrifice, blood samples were collected by cardiac puncture, placed in tubes, and immediately centrifuged at 1000 × *g* for 10 min. Then the serum thyroid hormone concentration was estimated by chemiluminescence using the Immunolite 2000 kit (Biomedical Technologies Inc., Strougerton, MA, USA) at Weinmann Clinical Analysis Laboratory.

2.5. Tert-butyl hydroperoxide-initiated chemiluminescence

Chemiluminescence (CL) was measured in a liquid scintillation counter in the out-of-coincidence mode (LKB Rack Beta Liquid Scintillation Spectrometer 1215, LKB – Produkter AB, Sweden). Homogenates were placed in low-potassium vials at a protein concentration of 0.5–1.0 mg/mL in a reaction medium consisting of 120 mmol/L KCl, 30 mmol/L phosphate buffer (pH 7.4). Measurements were started by the addition of 3 mmol/L tert-butyl hydroperoxide and data expressed as counts per second per milligram of protein of the homogenates (cps/mg protein) (Gonzalez Flecha et al., 1991).

2.6. Thiobarbituric acid reactive substances method (TBARS)

For the TBARS assay, trichloroacetic acid (10%, w/v) was added to the homogenate to precipitate proteins and to acidify samples (Buege and Aust, 1978). This mixture was then centrifuged (1000 × *g*, 3 min). The protein-free sample was extracted and thiobarbituric acid (0.67%, w/v) was added to the reaction medium. Tubes were placed in a water bath (100 °C) for 15 min. Absorbency was read at 535 nm in a spectrophotometer. Commercially available malondialdehyde was used as a standard. Results were expressed as micromoles per milligram of protein.

2.7. Carbonyl assay

Tissue samples were incubated with 2,4 dinitrophenylhydrazine (DNPH 10 mmol/L) in 2.5 mol/L HCl solution for 1 h at room temperature, in the dark. Samples were vortexed every 15 min. Then 20% TCA (w/v) solution was added in tube samples, left in ice for 10 min and centrifuged for 5 min at 1000 × *g*, to collect protein precipitates. Another wash was performed with 10% TCA. The pellet was washed three times with ethanol:ethyl acetate (1:1) (v/v). The final precipitates were dissolved in 6 mol/L guanidine hydrochloride solution, left for 10 min at 37 °C, and read at 360 nm (Reznick and Packer, 1994). The results were expressed as nmol/mg protein.

2.8. Determination of oxidized and reduced glutathione concentration

To determine total glutathione (expressed as mmol/mg protein), tissue was deproteinized with 2 mol/L perchloric acid, centrifuged for 10 min at 1000 × *g* and supernatant was neutralized with 2 mol/L potassium hydroxide. The reaction medium contained 100 mmol/L phosphate buffer (pH 7.2), 2 mmol/L nicotinamide dinucleotide phosphate acid, 0.2 U/mL glutathione reductase, 70 μmol/L 5,5′ dithiobis (2-nitrobenzoic acid). To determine reduced glutathione, the supernatant was neutralized with 2 mol/L potassium hydroxide, reacted with 70 μmol/L 5,5′ dithiobis (2-nitro benzoic acid), and read at 420 nm (Akerboom and Sies, 1981).

2.9. Determination of antioxidant enzyme activities

Superoxide dismutase (SOD) activity, expressed as units per milligram of protein, was based on the inhibition of superoxide radical reaction with pyrogallol (Marklund, 1985). Catalase (CAT) activity was determined by following the decrease in 240-nm absorption of hydrogen peroxide (H_2O_2). It was expressed as nanomols of H_2O_2 reduced per minute per milligram of protein (Aebi, 1984). Glutathione peroxidase (GPx) activity was measured by following NADPH oxidation at 340 nm as described by Flohé and Gunzler (1984). GPx results were expressed as nanomols of peroxide/hydroperoxide reduced per minute per milligram of protein. Glutathione-S-transferase (GST) activity, expressed as nanomols per milligram of protein, was measured by the rate of formation of dinitrophenyl-S-glutathione at 340 nm (Mannervik and Gluthemberg, 1981). Glutathione reductase (GR) activity was expressed as nanomols of GSSG reduced per minute per milligram of protein at 340 nm, as described by Carlberg and Mannervik (1985).

2.10. Western blot analysis

The tissue was homogenized (Tris 20 mmol/L, NaCl 150 mmol/L, EDTA 5 mmol/L, glycerol 10%, phenyl methyl sulphonyl fluoride (PMSF) 20 mmol/L, aprotinin 10 μL/mL and leupeptin 10 μL/mL) in Ultra-Turrax. The suspension was centrifuged at 1000 × *g* for 10 min at 0–4 °C to remove the nuclei and cell debris and supernatants were used for the assay. Thirty micrograms of protein

were subjected to one-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in a discontinuous system using 12% (w/v) separating gel and stacking gel (Laemmli, 1970). The proteins separated were transferred to nitrocellulose membranes electrophoretically using buffer pH 8.2, containing 20 mmol/L Tris, 150 mmol/L glycine, methanol 20% (v/v) SDS 0.1% (w/v), in a cooled Bio-Rad TransBlot unit. Then, non-specific protein-binding sites were blocked with 1 h incubation with non-fat milk in Tris-buffer. The membranes were processed for immunodetection using sheep anti-Cu/Zn SOD polyclonal antibodies, rabbit anti-GST polyclonal antibodies and rabbit anti-CAT polyclonal antibodies as primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). The bound primary antibodies were detected using rabbit anti-sheep or goat anti-rabbit horseradish peroxidase-conjugate secondary antibody and membranes were revealed for chemiluminescence. Autoradiographic films were quantitatively analyzed for the Cu/Zn SOD, GST and CAT protein levels with an image densitometer (Imagemaster VDS CI, Amersham Biosciences Europe, IT). The molecular weights of the protein bands were determined by reference to a standard molecular weight marker (RPN 800 rainbow full range Bio-Rad, CA, USA). The results from each membrane were normalized through Ponceau red method (Klein et al., 1995).

2.11. Determination of protein concentration

Protein was measured by the method of Lowry et al. (1951), using bovine serum albumin as standard.

2.12. Myocardial time course of oxidative stress

We have followed the oxidative stress time course in the myocardium from the first to the fourth week of treatment with thyroxine, evaluating antioxidant enzyme activities, redox status (GSH/GSSG ratio) and heart failure development by means LVSP and LVEDP.

2.13. Statistical analysis

Data were expressed as mean ± S.E.M. and compared using the unpaired Student's *t*-test. The correlation between two variables was analyzed by Pearson's correlation. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Hyperthyroidism and heart failure development

At the end of a 4-week post-treatment period, thyroxine serum levels were significantly higher in hyperthyroid (30%) rats than in controls (Table 1). A body weight loss (13%) was noted in hyperthyroid animals when compared to control. Thyroxine has induced cardiac hypertrophy as detected by the heart to body weight ratio, which was (60%) higher in the hyperthyroid group than in control ($P < 0.05$) (Table 1). The hyperthyroid state has induced hemodynamic changes, as observed by the elevation of LVSP (by 40%) and LVEDP (by 110%) at the fourth week of treatment as compared to control. Associated with these changes, it was observed lung and liver congestion (Table 1).

Table 1
Thyroid hormone levels, morphometric and hemodynamic parameters after 4-week treatment

Parameters	Control	Hyperthyroid
T_4 (ng/mL)	3.8 ± 0.1	24.5 ± 0.7*
Body weight (g)	230 ± 25	199 ± 18*
Heart weight (g)	0.68 ± 0.1	0.95 ± 0.1*
Heart/body weight ($\times 10^3$ mg/g)	3.0 ± 0.02	4.8 ± 0.05*
LVSP (mmHg)	128 ± 11	185 ± 10*
LVEDP (mmHg)	4.9 ± 0.7	11 ± 1.3*
Lung/body weight ($\times 10^3$ mg/g)	0.7 ± 0.1	1.1 ± 0.3*
Liver/body weight ($\times 10^3$ mg/g)	3.3 ± 0.4	5.0 ± 0.9*

Values are expressed as mean ± S.E.M., of 10 animals per group.
* Significantly different from control ($P < 0.05$).

Table 2
Myocardial markers of oxidative damage to membrane lipids (chemiluminescence and TBARS) and proteins (carbonyl groups) after 4-week treatment

Parameters	Control	Hyperthyroid
Chemiluminescence (cps/mg protein 10^5)	167 ± 14	220 ± 33*
TBARS (μmol/mg protein)	2.9 ± 0.4	4.3 ± 0.6*
Carbonyl groups (nmol/mg protein)	2.9 ± 0.1	4.1 ± 0.5*

Values are expressed as mean ± S.E.M., of 10 animals per group.
* Significantly different from control ($P < 0.05$).

3.2. Oxidative damage

The changes in oxidative damage markers are summarized in Table 2. The state of hyperthyroidism caused a significant elevation in lipid peroxidation products, as indicated by CL (32%) as well as TBARS (48%) in cardiac tissue compared to control ($P < 0.05$). The L-thyroxine treatment also resulted in increased myocardial oxidative damage to proteins measured using the carbonyl assay (41%) as compared to controls ($P < 0.05$).

3.3. Glutathione metabolism

It has also been shown that the myocardial status of glutathione metabolism in the hyperthyroid group was changed at the fourth week of treatment. There was a 46% and 21% decrease in the reduced and total GSH, respectively, in hyperthyroid animals as compared to the control. The redox status (GSH/GSSG ratio), an oxidative stress indicator, was found to be significantly reduced (82%) in hyperthyroid rats ($P < 0.05$) (Table 3). This parameter was negatively correlated with LVEDP ($r = -0.94$, $P < 0.001$).

3.4. Antioxidant enzyme activities and concentration

The L-thyroxine treatment of rats resulted in the elevation of SOD activity, which was 31% higher in hyperthyroid animals

Table 3
Status of glutathione metabolism in control and hyperthyroid rat hearts, after 4-week treatment (GSH: reduced glutathione; GSSG: glutathione disulfide)

Parameters	Control	Hyperthyroid
GSH (nmol/mg protein)	1.06 ± 0.10	0.57 ± 0.09*
GSSG (nmol/mg protein)	0.11 ± 0.03	0.41 ± 0.18*
GSH/GSSG (redox status)	10.8 ± 2.0	1.84 ± 0.7*
Total GSH (nmol/mg protein)	1.23 ± 0.1	0.97 ± 0.16*

Values are expressed as mean ± S.E.M., of 5 animals per group.
* Significantly different from control ($P < 0.05$).

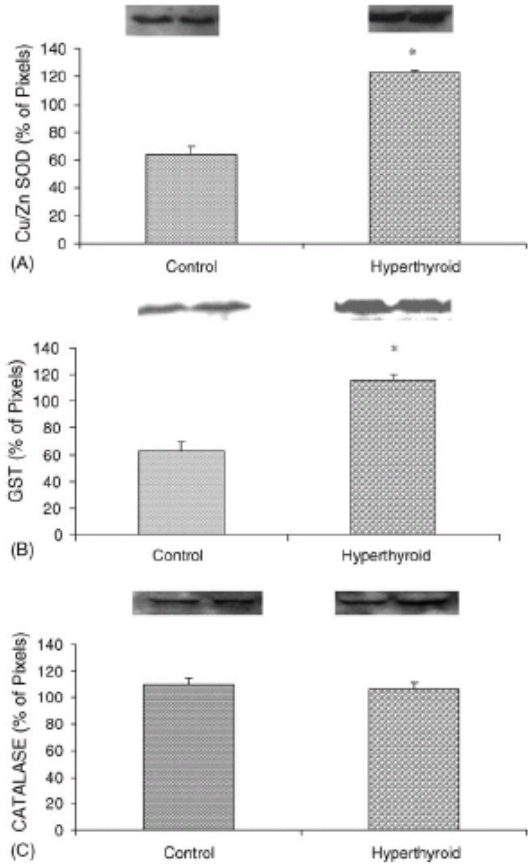


Fig. 1. Western blot analysis in cardiac homogenates using Cu/Zn SOD antibody (A), GST antibody (B) and CAT antibody (C). (*) Significantly different from the control ($P < 0.05$). Data as mean ± S.E.M. from 8 animals in each group and five experiments.

than in controls ($P < 0.05$). No significant change was observed in CAT activity in L-thyroxine-treated animals. In hyperthyroid animals GPx activity was significantly increased (61%) as compared to the control. Hyperthyroidism has also induced an increase in GR (39%), and GST (60%), activities as compared to the control (Fig. 2A–C). In order to examine the molecular mechanism responsible for changes in the activities of these enzymes, protein levels of antioxidant enzymes were measured at the end of a 4-week post-treatment period by Western blot. The protein levels of Cu/Zn SOD and GST have shown an elevation, in hyperthyroid rat heart (87% and 84%, respectively) as compared to the control (Fig. 1A and B). CAT concentration was not significantly different between groups ($P < 0.05$) (Fig. 1C). Cu/Zn SOD protein levels are positively correlated with CL ($r = 0.80$, $P < 0.05$), TBARS ($r = 0.87$, $P < 0.05$) and carbonyl groups ($r = 0.85$, $P < 0.05$). The protein levels of GST have also shown a significant positive correlation with lipid peroxidation: CL ($r = 0.80$, $P < 0.05$), TBARS ($r = 0.92$, $P < 0.05$), as well as with protein oxidation ($r = 0.95$, $P < 0.05$).

3.5. Myocardial time course of oxidative stress

We observed that oxidative stress (evaluated by GSH/GSSG) was increased since the first week of treatment with thyroxine. The lowest values of GSH/GSSG ratio were observed at the second week of hyperthyroidism (Fig. 2A). Myocardial oxidative stress increment was accompanied by the elevation in the antioxidant enzyme activities that were higher in hyperthyroid animals than in controls, except for catalase which was unchanged (Fig. 2A–C). The highest activities were observed at the second week of thyroxine treatment. Left ventricular function impairment was, however, more pronounced at the fourth week of hyperthyroidism (Fig. 2D).

4. Discussion

Our results show that a chronic hyperthyroid state was successfully achieved as observed from the elevation in serum T_4, reduction in body weight, and higher heart/body weight ratio in the thyroxine-treated animals. These findings are in accordance with literature using the same experimental protocol applied in this study (Ladenson et al., 1986). In this model it was also observed, at the fourth week of hyperthyroidism, congestive heart failure, characterized by LVEDP elevation and lung and liver congestion. Some evidence is provided in the literature demonstrating the involvement of ROS in intracellular signaling pathways that mediate hypertrophy and its progression to heart failure (Sabri et al., 2003).

The elevation of oxidative stress is well established in the hyperthyroid state in many different tissues. However, there is no study showing a more complete scenario of the oxidative stress in terms of damage to biomolecules, correlating these changes to protein expression of antioxidant enzymes. In this study, we observed an intense myocardial lipid peroxidation in hyperthyroid animals demonstrated by TBARS and CL assessments. Clinical studies have found increased lipid peroxidation through higher TBARS concentration and chemiluminescence in the urine and serum of hyperthyroid patients (Abalovich et al., 2003). In experimental hyperthyroidism increased lipid peroxidation and protein oxidation were also demonstrated in different tissues (Fernández et al., 1985; Fernández and Videla, 1993; Tapia et al., 1999; Goswami et al., 2003). Enhanced myocardial protein oxidation was also shown in this study by means of carbonyl group measurement. An elevation of this protein oxidation marker was demonstrated in the plasma of hyperthyroid patients (Venditti et al., 2003; Goswami et al., 2003). Thus, our results have shown an increase in oxidative damage to myocardial lipids and proteins. Such an effect may be related to the enhanced metabolic rate induced by thyroid hormone administration, leading to an accelerated ROS production (Wilson et al., 1989; Seven et al., 1996).

In order to evaluate the other side of the oxidative stress balance, we have also examined the non-enzymatic antioxidant reserve of the myocardium. The hyperthyroid group in our study has shown higher GSSG and lower GSH levels than the control, suggesting an oxidative stress situation in the myocardium. Glutathione has been considered one of the major hydrosoluble

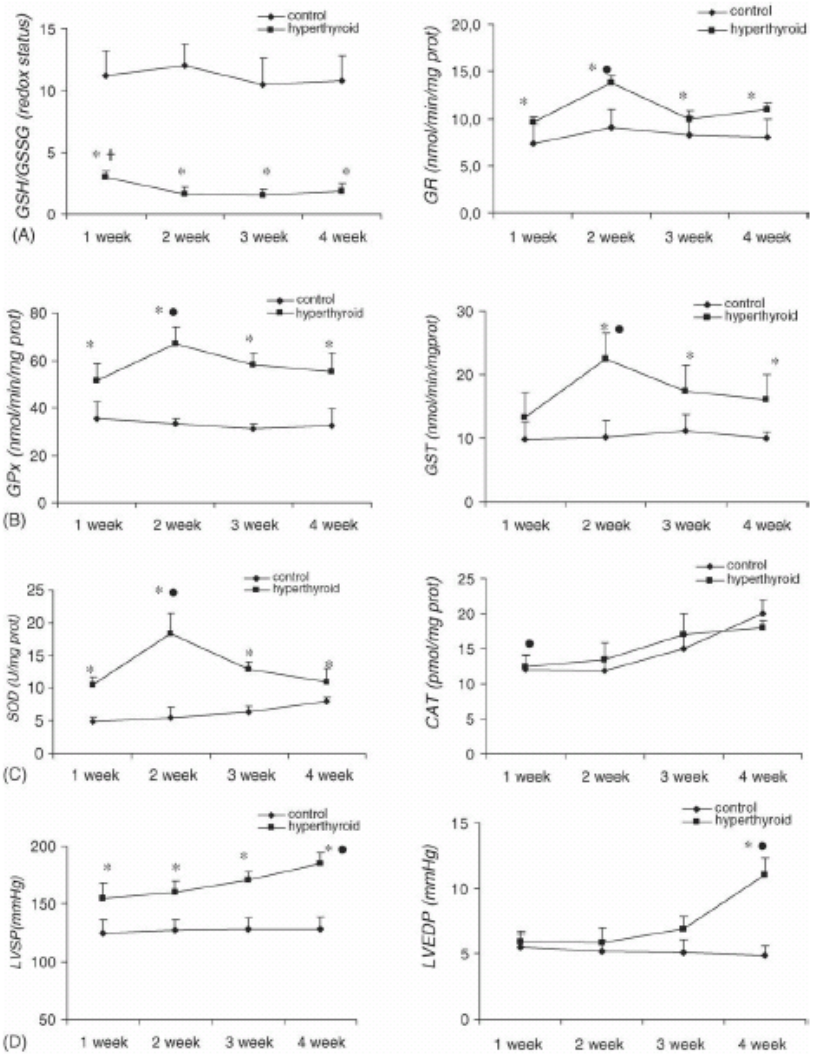


Fig. 2. Oxidative stress and hemodynamic time course profile in the cardiac homogenates from the first to the fourth week of treatment with thyroxine, by means the determination of GSH/GSSG ratio, and antioxidant enzyme activities of GRx (A), GPx and GST (B), SOD and CAT (C), LVSP and LVEDP (D). Data as mean ± S.E.M. from 8 animals in each group. (*) Significantly different from the control ($P < 0.05$). (●) Significantly different from the other time points in the hyperthyroid groups ($P < 0.05$). (†) Significantly different from the week 1 in relation to the other time points in the hyperthyroid groups ($P < 0.05$).

cellular antioxidants, detoxificating peroxides/hydroperoxides. GSH/GSSG ratio may indicate the oxidative stress status of cells (Dincer et al., 2002). This parameter was strongly correlated with myocardial dysfunction (LVEDP elevation), reinforcing the role of oxidative stress in heart failure induced by hyperthyroidism.

In terms of antioxidant enzyme activities, no changes were found in CAT activity and concentration in the myocardium of hyperthyroid rats. SOD activity, however was found to be increased in the hyperthyroid animals as compared to controls. Since SOD is specific to superoxide radicals detoxification, a likely assumption is that this ROS concentration is increased in heart tissue, especially at the second week where this enzyme activity is the highest. Myocardial expression of Cu/Zn SOD was also enhanced in the hyperthyroid group. Superoxide radicals levels probably modulate regulation of this enzyme. Das and Chainy (2004) have demonstrated the specific influence of ROS in the modulation of antioxidant enzyme activities. GPx is the main enzymatic mechanism for the disposal of peroxides in cardiac tissue, producing water or alcohol and GSSG. Regen-

eration of GSH from GSSG in the cell is catalyzed by the GR enzyme (Mannervik and Gluthemberg, 1981; Li et al., 2000). Augmented activity of this enzyme reflects intense participation of GSH in blocking the peroxidation process, especially at the second week. The augmented activity and protein expression of GST found in this study can also reflect the important role of the ROS. GST is a very important enzyme in the detoxification of xenobiotics and, therefore, could be involved in L-thyroxine metabolism. The elevation in GST activity and concentration also indicates that an oxidative stress condition is taking place, since it is considered an oxidative stress marker (Neefjes et al., 1999). Oxidative stress time course analysis revealed that all the antioxidant enzyme activities, except catalase, are increased in the second week of thyroxine treatment, suggesting that ROS production is the highest at this time point. Coincidently, GSH concentration is the lowest in the second week of hyperthyroidism. These adaptations may maintain ventricular function partially preserved at this time point, but it becomes to be seriously impaired from the third to the fourth week after treatment.

We demonstrated a strong positive correlation between antioxidant enzyme concentration, and lipid peroxidation/carbonylation. These data indicate that could have modulation ROS-mediated of antioxidant enzyme activity protein expression. Gene expression is controlled by a complex mechanism, whose regulation is mainly at the transcriptional level, although its regulation could occur during postranscriptional process, at the translation level and postranslation. Nevertheless, gene expression regulatory mechanism was not the purpose of this work.

In summary, thyroid hormone elevation stimulates the metabolic rate, possibly leading to ROS generation. These ROS increase cause oxidative damage to lipids and proteins, and could activate redox-sensitive pathways of protein concentration, promoting an elevation of antioxidant enzyme activity and protein expression. As a consequence, there is a depletion of reduced glutathione content utilized in the ROS detoxification process. This redox state imbalance might reinforce redox-sensitive signaling pathways. In terms of oxidative stress time course profile, intense biochemical changes occur at the second week of hyperthyroidism and it may determine the hemodynamic changes observed later at the fourth week of treatment. This is a pioneering study in demonstrating an association between concentration and antioxidant enzyme activity in the hyperthyroid failing rat heart. The measurements used in this work could be useful as oxidative stress markers in patients, since they can be evaluated in systemic blood (Repetto et al., 1996). Thus antioxidant therapy emerges as an attractive approach in the heart failure treatment.

Acknowledgements

CNPq and FAPERGS, Brazilian Research Agencies supported this study. We would like to thank the Weinmann Clinical Analysis Laboratory for performing the thyroid hormone measurements.

References

Abalovich, M., Llesuy, S., Gutierrez, S., Repetto, M., 2003. Peripheral parameters of oxidative stress in Graves' disease: the effects of methimazole and 131 iodine treatments. Clin. Endocrinol. 59, 321–327.
Aebi, H., 1984. Catalase in vitro. Meth. Enzymol. 105, 121–126.
Akerboom, T., Sies, H., 1981. Assay glutathione disulfide and glutathione mixed disulfides in biological samples. Meth. Enzymol. 77, 373–382.
Asayama, K., Kato, K., 1990. Oxidative muscular injury and its relevance to hyperthyroidism. Free Rad. Biol. Med. 8 (3), 293–303.
Buege, J.A., Aust, S.D., 1978. Microsomal lipid peroxidation. Meth. Enzymol. 52, 302–309.
Carlberg, I., Mannervik, B., 1985. Glutathione transferase. Meth. Enzymol. 113, 484–489.
Das, K., Chainy, G.B.N., 2001. Modulation of rat liver mitochondrial antioxidant defence system by thyroid hormone. Biochim. Biophys. Acta 1537, 1–13.
Das, K., Chainy, G.B.N., 2004. Thyroid hormone influences antioxidant defense system in adult rat brain. Neurochem. Res. 29, 1755–1766.
Dincer, Y., Akca, T., Alademir, Z., Ilkova, H., 2002. Effect of oxidative stress on glutathione pathway in red blood cells from patients with insulin dependent diabetes mellitus. Metabolism 51, 1360–1362.
Esterbauer, H., Shaur, R.J., Zollner, H., 1991. Chemistry and Biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Rad. Biol. Med. 11, 81–128.
Fernández, V., Videla, L., 1993. Influence of hyperthyroidism on superoxide radical and hydrogen peroxide production by rat liver submitochondrial particles. Free Rad. Res. Comm. 18, 329–335.
Fernández, V., Barrientos, X., Kipreos, K., 1985. Superoxide radical generation, NADPH oxidase activity, and cytochrome P-450 content of rat liver microsomal fractions in an experimental hyperthyroid state: relation to lipid peroxidation. Endocrinology 117, 496–501.
Flohé, L., Gunzler, W.A., 1984. Assay of glutathione peroxidase. Meth. Enzymol. 105, 14–121.
Gonzalez Flecha, B., Llesuy, S., Boveris, A., 1991. Hydroperoxide-initiated chemiluminescence: an assay for oxidative stress in biopsies of liver, heart and muscle. Free Rad. Biol. Med. 10, 41–47.
Goswami, K., Nandakumar, D.N., Koner, B.C., 2003. Oxidative changes and desialylation of serum proteins in hyperthyroidism. Clin. Chim. Acta 337, 163–168.
Guerrero, A., Pamplona, R., Portero-Otin, M., Barja, G., Lopez-Torres, M., 1999. Effect of thyroid status on lipid composition and peroxidation in the mouse liver. Free Rad. Biol. Med. 26, 73–80.
Hogg, N., Kalyanamaran, B., 1999. Nitric oxide and lipid peroxidation. Biochim. Biophys. Acta 1411, 378–384.
Klein, I., 1990. Thyroid hormone and the cardiovascular system. Am. J. Med. 88, 631–640.
Klein, D., Kern, R.M., Sokol, R.Z., 1995. A method for quantification and correction of proteins after transfer to immobilization membranes. Biochem. Mol. Biol. 36, 1.
Ladenson, P.W., Kieffer, J.D., Farwell, A.P., Ridgway, E.C., 1986. Modulation of myocardial L-triiodthyronine receptors in normal, hypothyroid and hyperthyroid rats. Metabolism 35, 5–12.
Laemmli, V., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227, 680–685.
Li, T., Danelisen, I., Belló-Klein, A., Singal, P.K., 2000. Effects of probucol on changes of antioxidant enzymes in adriamycin induced cardiomyopathy in rats. Cardiovasc. Res. 46, 523–530.
Llesuy, S.F., Milei, J., Molina, H., Boveris, A., Milei, S., 1985. Comparison of lipid peroxidation and myocardial damage induced by adriamycin and 4′-epiadrimycin in mice. Tumori 71, 241–249.
Lowry, O.H., Rosebrough, A.L., Farr, A.L., Randall, R., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275.
Mannervik, B., Gluthemberg, C., 1981. Glutathione transferase. Meth. Enzymol. 77, 231–235.
Mano, T., Sinohara, R., Sawai, Y., 1995. Effects of thyroid hormone on coenzyme Q and other free radical scavengers in rat heart muscle. J. Endocrinol. 145, 131–136.

Marklund, S., 1985. Handbook of Methods for Oxygen Radical Research. CRC Press, Boca Raton, pp. 243–247.

Neefjes, V.M., Evelo, C.T., Baar, L.G., Blanco, C.E., 1999. Erythrocyte glutathione S transferase as a marker of oxidative stress at birth. Arch. Dis. Child Fetal Neonat. 81 (2), F130–F133.

Repetto, M., Reides, C., Carretero, M.L.G., Costa, M., Griemberg, Llesuy, S., 1996. Oxidative stress in blood of HIV infected patients. Clin. Chim. Acta 255, 107–177.

Reznick, A.Z., Packer, L., 1994. Carbonyl assay for determination of oxidatively modified proteins. Meth. Enzymol. 233, 357–363.

Sabri, A., Hughie, H.H., Lucchesi, P.A., 2003. Regulation of hypertrophic and apoptotic signaling pathways by reactive oxygen species in cardiac myocytes. Antiox. Redox. Signal. 5, 731–740.

Seven, A., Seymen, O., Hatemi, S., Hatemi, H., Yigit, G., Candan, G., 1996. Antioxidant status in experimental hyperthyroidism: effect of Vitamin E supplementation. Clin. Chim. Acta 256, 65–73.

Singal, P.K., Iliskovic, N., 1998. Doxorubicin-induced cardiomyopathy. N. Engl. J. Med. 339, 900–905.

Tapia, G., Comejo, P., Fernandez, V., Videla, L.A., 1999. Protein oxidation in thyroid hormone-induced liver oxidative stress: relation to lipid peroxidation. Toxicol. Lett. 106, 209–241.

Venditti, P., Di Meo, S., De Leo, T., 1996. Effect of thyroid state on characteristics determine the succeptibility to oxidative stress of mitochondrial fractions from rat liver. Cell. Physiol. Biochem. 6, 283–295.

Venditti, P., De Rosa, R., Di Meo, S., 2003. Effect of thyroid state on H_2O_2 production by rat liver mitochondria. Mol. Cell. Endocrinol. 205, 185–192.

Wilson, R., Chopra, M., Bradley, H., Mckillop, J., Smth, We., Thomson, J.A., 1989. Free radicals and Graves' disease: effect of therapy. Clin. Endocrinol. 30, 429–433.

6.2 Artigo 2*: Oxidative stress activates insulin-like growth factor I receptor protein expression mediating cardiac hypertrophy induced by thyroxine (Submetido para: Molecular and Cellular Biochemistry)*

Oxidative stress activates insulin-like growth factor I receptor protein expression, mediating cardiac hypertrophy induced by thyroxine

Araujo, A.S.R.[1]; Enzveiler, A. T.[1]; Schenkel, P.[1]; Fernandes, T.R.G[1]; Ribeiro M.F.M[1]; Partata, W.A.[1]; Llesuy, S[2, 3], Belló-Klein, A[1].

[1]*Departamento Fisiologia, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre - Brazil*

[2] *Cátedra de Química General y Inorgánica, Facultad de Farmácia y Bioquímica, Universidad de Buenos Aires - Argentina*

[3] *Departamento de Fisiologia e Farmacologia, Universidade Federal de Santa Maria, Santa Maria - Brazil*

Corresponding author: Dr. Adriane Belló-Klein

Rua Sarmento Leite, 500.

CEP: 90050-170 - Porto Alegre - RS - Brazil

Email: belklein@ufrgs.br

Telephone: 552151 33163621 Fax: 552151 33163656

Abstract

Thyroxine can cause cardiac hypertrophy by activating growth factors. Since oxidative stress is enhanced in the hyperthyroidism, it would control protein expression involved in this hypertrophy. Male Wistar rats were divided into: control, vitamin E (20 mg/kg/day i.p.), hyperthyroid (thyroxine 12 mg/L, in drinking water), and hyperthyroid+vitamin E. After four weeks the contractility index of left ventricle, and cardiac mass were increased and the relaxation index was decreased in hyperthyroid group. An increase in lipid peroxidation, and a decrease in total glutathione was induced by thyroxine and avoided by vitamin E, a classical antioxidant, administration. Superoxide dismutase (SOD) and glutathione-S-transferase (GST) activities were increased in hyperthyroid and vitamin E avoided changes in SOD. Protein expression of SOD, GST, and insulin-like growth factor-I receptor (IGF-IR) were increased by thyroxine, and vitamin E promoted a significant reduction in SOD and IGF-IR expression. These results indicate that oxidative stress is involved in cardiac hypertrophy, and suggest a role for IGF-IR as a mediator for this adaptive response in experimental hyperthyroidism.

***Key words**: hyperthyroidism*, *glutathione metabolism, heart failure, protein expression, vitamin E, IGF-IR.*

1. Introduction

Thyrotoxic cardiomyopathy is associated with thyroid hormones effects in the cardiovascular system [1]. The thyroid hormones may cause hemodynamic changes, including an increase in myocardial contractility, an elevation in cardiac output, a widening of pulse pressure, cardiac arrhythmias, and atrial fibrillation [2]. The effects of thyroid hormones have been attributed to the nuclear receptor-mediated regulation of transcription. Nevertheless, increased oxygen demand to myocardium, provided by hyperthyroidism, can result in the production of reactive oxygen species (ROS) [1]. ROS can play an important role in mediating the effects of thyroid hormones, especially those related to electrical and mechanical activity of the heart [3].

In order counteract ROS production, the enzymatic and nonenzymatic antioxidant defense systems are present to minimize the oxidative damage due to these species [4]. However, when ROS generation is exarcebated and overcome the antioxidant capacity of cells, oxidative stress develops, leading to potential tissue damage [5]. Evidence from experimental as well as clinical studies point out the involvement of oxidative stress in the pathogenesis of cardiac dysfunction, such as cardiac hypertrophy and failure [3]. There is a positive correlation between hyperthyroidism and cardiac hypertrophy [6], and ROS may be involved in this adaptive answer [7]. However, the molecular mechanisms of ROS mediating these effects in myocardium are still poorly understood.

In a previous study, we have demonstrated a positive correlation among oxidative stress, cardiac hypertrophy, and left ventricular diastolic dysfunction in experimental hyperthyroidism, indicating that ROS may contribute to the progression to heart failure in this model [8]. In hyperthyroidism, however, there is scarce information about the induction of gene expression of redox-sensitive factors in the hypertrophic heart, including insulin-like growth factor-I receptor (IGF-IR), a critical factor for muscle cell growth [9]. Insulin-like growth factor I (IGF-I) plays an essential role in the regulation of cellular growth and development [10]. There is experimental evidence for the role of IGF-I in the initiation and development of myocardial hypertrophy [11]. Thyroid hormones are involved in the regulation of gene expression, and may modulate IGF-IR expression, and, thus, it could interfere in induced-thyroxine cardiac hypertrophy.

In the present study, we hypothesized that vitamin E treatment would decrease oxidative stress, blocking redox-regulated pathways involved in the cell growth, such as those induced by IGF-IR. Thus, it would avoid the development of myocardial hypertrophy and failure in experimental hyperthyroidism.

2. Material and Methods

2.1. Animals

Forty male Wistar rats (200±20 g) were obtained from the Central Animal House of the Universidade Federal do Rio Grande do Sul, Brazil. Animals were housed in plastic cages (five animals each) and received water and pelleted food *ad libitum*. They were maintained under standard laboratory conditions (controlled temperature of 21°C, 12 hours light/dark cycle). Animals were weekly weighed to follow body weight gain, during the experimental protocol (28 days). They were divided into four groups (n=10/group): a) control, that received water *ad libitum* and subcutaneous injections of mineral oil; b) vitamin E, that received subcutaneous injections of vitamin E (20 mg/Kg/day, in mineral oil) [12]; c) hyperthyroid, which received L-thyroxine (T_4) (12 mg/L in drinking water); d) hyperthyroid+vitamin E, which received T_4 and vitamin E, at the same conditions as above described [13].

2.2. Hemodynamic measurements and cardiac hypertrophy development

Cardiac hemodynamics was assessed at the end of the fourth week of treatment. In brief, rats were anesthetized (ketamine 90mg/kg; xylazine 10mg/kg, i.p.) and the right carotid artery was cannulated with a PE 50 catheter connected to a strain gauge transducer (Narco Biosystem Pulse Transducer RP-155, Houston, Texas, USA) linked to a pressure amplifier (HP 8805C, Hewlett

Packard, USA). Pressure readings were taken in a microcomputer equipped with an analogue-to-digital conversion board (CODAS 1kHz sampling frequency, Dataq Instruments, Inc., Akron, Ohio, USA). The catheter was advanced into left ventricle (LV) for recording the left ventricular systolic pressure (LVSP, mmHg), the left ventricular end diastolic pressure (LVEDP, mmHg), and the positive and negative pressure derivatives (±d*P*/d*t*). Positive derivative of ventricular pressure is considered as the contractility index and the negative derivative is the relaxation index. The cardiac hypertrophy was evaluated by heart weight (in mg) to body weight (in g) ratio.

2.3. Tissue preparation

Four weeks after treatment, rats were decapitated and the hearts were rapidly excised, weighed, and homogenized (1.15% w/v KCl and phenyl methyl sulphonyl fluoride PMSF 20 mmol/L) in Ultra-Turrax. The suspension was centrifuged at 600 g for 10 min at 0-4°C to remove the nuclei and cell debris [14] and supernatants were used for the assay of lipid peroxidation and enzymatic activity. In the moment of the sacrifice, cardiac tissue samples were also rapidly removed and frozen at -80°C, for the evaluation of glutathione content and protein expression, and homogeneized as described at “Determination of total glutathione content” and “Western blot analysis” sections, respectively. Lung and liver were also taken and weighed in order to estimate these organs congestion. These parameters were expressed as organs wet weight (in mg) to body weight (in g) ratio.

2.4. Thyroid hormones concentration

Blood samples were collected by cardiac punction, and immediately centrifuged at 1000 g for 10 min. After, serum thyroid hormones concentration was estimated by chemiluminescence using the Immunolite 2000 kit (Biomedical Technologies, Inc., Strougerton, MA, USA) at Weinmann Clinical Analysis Laboratory.

2.5. Tert-butyl hydroperoxide-initiated chemiluminescence

Chemiluminescence (CL) was measured in a liquid scintillation counter in the out-of-coincidence mode (LKB Rack Beta Liquid Scintillation Spectrometer 1215, LKB – Produkter AB, Sweden). Homogenates were placed in low-potassium vials at a protein concentration of 0.5-1.0 mg/mL in a reaction medium consisting of 120 mmol/L KCl, 30 mmol/L phosphate buffer (pH=7.4). Measurements were started by the addition of 3 mmol/L tert-butyl hydroperoxide and data expressed as counts per second per milligram of protein of the homogenates (cps/mg protein) [15].

2.6. Thiobarbituric acid reactive substances method (TBARS)

For the TBARS assay, trichloroacetic acid (10% w/v) was added to the homogenate to precipitate proteins and to acidify samples [16]. This mixture was then centrifuged (1000 g, 3 min). The protein-free sample was extracted and

thiobarbituric acid (0.67% w/v) was added to the reaction medium. Tubes were placed in a water bath (100°C) for 15 min. Absorbency was read at 535 nm in a spectrophotometer. Commercially available malondialdehyde was used as a standard. Results were expressed as micromoles per milligram of protein.

2.7. Determination of total glutathione content

To determine total glutathione content, tissue was deproteinized with 2 mol/L perchloric acid, centrifuged for 10 min at 1000 g, and the supernatant was neutralized with 2 mol/L potassium hydroxide. The reaction medium contained 100 mmol/L phosphate buffer (pH 7.2), 2 mmol/L nicotinamide dinucleotide phosphate acid, 0.2 U/mL glutathione reductase, 70 µmol/L 5,5' dithiobis (2-nitro benzoic acid), and was measured at 420 nm. The results were expressed in nmol per milligram of protein [17].

2.8. Determination of antioxidant enzyme activities

Superoxide dismutase (SOD) activity, expressed as units per milligram of protein, was based on the inhibition of superoxide radical reaction with pyrogallol [18]. Catalase (CAT) activity was determined by following the decrease in 240-nm absorption of hydrogen peroxide (H_2O_2). It was expressed as nanomol of H_2O_2 reduced per minute per milligram of protein [19]. Glutathione-S-transferase (GST) activity, expressed as nanomol per milligram of protein, was measured by the rate of formation of dinitrophenyl-S-glutathione at 340nm [20].

2.9. Western Blot Analysis

The tissue was homogenized (Tris 20 mmol/L, NaCl 150 mmol/L, EDTA 5 mmol/L, glycerol 10%, phenyl methyl sulphonyl fluoride-PMSF 20 mmol/L, aprotinin 10μL/mL and leupeptin 10μL/mL) in Ultra-Turrax. The suspension was centrifuged at 1000 g for 10 min at 0-4°C to remove the nuclei and cell debris and supernatants were used for the assay. Thirty micrograms of protein were subjected to one dimensional sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) in a discontinuous system using 12% (w/v) separating gel and stacking gel [21]. The protein separated were transferred to nitrocellulose membranes electrophoretically using buffer pH 8.2, containing 20 mmol/L Tris, 150 mmol/L glycine, methanol 20%(V/V) SDS 0.1% (w/v), in a cooled Bio-Rad TransBlot unit. After, non-specific protein-binding sites were blocked with 1 h incubation with non-fat milk in Tris-buffer. The membranes were processed for immunodetection using rabbit anti-CAT polyclonal antibody (65 kDa), sheep anti-Cu/Zn SOD polyclonal antibody (23 kDa), rabbit anti-GST polyclonal antibody (26 kDa), and rabbit anti-IGF-IR polyclonal antibody (98 kDa) as primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). The bound primary antibodies were detected using rabbit anti-sheep or goat anti-rabbit horseradish peroxidase-conjugate secondary antibodies and membranes were revealed for chemiluminescence. The autorradiographs generated were quantitatively analyzed for the CAT, Cu/Zn SOD, GST, and IGF-IR protein levels with an image densitometer (Imagemaster VDS CI, Amersham Biosciences Europe,IT). The molecular weights of the bands were determined by reference to

a standard molecular weight marker (RPN 800 rainbow full range Bio-Rad, CA, USA). The results from each membrane were normalized through Ponceau red method [22].

2.10. Determination of Protein Concentration

Protein was measured by the method of Lowry [23], using bovine serum albumin as standard.

2.11. Statistical analysis

Data were expressed as mean ± SDM and compared using one way ANOVA complemented with Student-Newmann-Keuls. The correlation between two variables was analyzed by Pearson's correlation. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Hyperthyroidism and Cardiac Remodeling

At the end of 4 week post-treatment period, thyroxine serum levels (in ng/mL) were significantly higher in hyperthyroid (24.0±0.7) and hyperthyroid+vitamin E (19.8±0.8) than control (3.8±0.1) and vitamin E (3.9±0.2). However, no statistical difference was found between the two hyperthyroid groups. It was noticed a body weight loss (13%) in hyperthyroid animals when

compared to control, and vitamin E administration avoided this change (Table 1). Thyroxine has induced cardiac hypertrophy as detected by heart to body weight ratio (mg/g), which was 60% higher in hyperthyroid group than control. However, after treatment with vitamin E, heart to body weight ratio in hyperthyroid+vitamin E group has shown a 23% reduction as compared to hyperthyroid and it is not different from control ($P<0.05$) (Table 1). In terms of intraventricular pressure, hyperthyroidism induced elevation of left ventricular systolic pressure (LVSP) and left ventricular end diastolic pressure (LVEDP) by 44% and 128%, respectively, as compared to control. LVSP was reduced by 20% in hyperthyroid group that received vitamin E treatment, but no significant decrease in LVEDP was observed with vitamin E treatment (Table 1). The positive derivative (+dP/dt) increased by 54%, and negative derivative ($-dP/dt$) decreased by 60% in the hyperthyroid. However, vitamin E administration, in the hyperthyroid animals, decreased $+dP/dt$ by 23%, as well as $-dP/dt$ by 28% (Table 1).

3.2. Oxidative damage to lipids

Changes in markers of lipid oxidative damage are summarized in Figure 1. Hyperthyroidism state caused a significant elevation in lipid peroxidation products, as indicated by chemiluminescence (32%) and TBARS (47%) in cardiac tissue as compared to control ($P<0.05$) (Figures 1A and 1B). However, in hyperthyroid+vitamin E group CL and TBARS were reduced by 25% and 33%, respectively, as compared to hyperthyroid ($P<0.05$). It was found that hypertrophy index showed a significant positive correlation with CL ($r=0.73$, $P<0.05$) and TBARS values ($r=0.8$, $P<0.05$).

3.3. Total glutathione content

It has also been shown that myocardial glutathione (GSH) content in the hyperthyroid group was changed. There was a 20% decrease in the total GSH, in hyperthyroid as compared to control ($P<0.05$) (Table 2). There was a restablishment of these values with vitamin E treatment in hyperthyroid rats, but they were still minor than vitamin E group. Vitamin E administration increased total GSH content in euthyroid animals ($P<0.05$) (Table 2).

3.4. Antioxidant enzyme activities and protein expression

The L-thyroxine treatment to rats resulted in no significant change in CAT activity (Table 2) and protein expression (Figure 2A) among the experimental groups. There was elevation of SOD activity in the hyperthyroid group, which was 83% higher than control (Table 2). The protein levels, evaluated by Western blot, of Cu/Zn SOD were elevated by 87% in hyperthyroid group as compared to control ($P<0.05$) (Figure 2B). The treatment of hyperthyroid animals with vitamin E reduced SOD activity by 36% (Table 2), and protein expression decreased by 16% in comparison to hyperthyroid (Figure 2B). Hyperthyroidism has also induced an increase in GST activity (Table 2) and protein expression (Figure 2C) (by 54% and 84%, respectively) as compared to control ($P<0.05$). The hyperthyroid+vitamin E group showed augmented GST activity as compared to vitamin E (82%) (Table 2, Figure 2C). No difference was found among vitamin E, hyperthyroid and hyperthyroid+vitamin E groups in terms of GST protein levels

(Figure 2C). In the hyperthyroid+vitamin E group the GST activity was additionaly increased in comparison to hyperthyroid (Table 2).

3.5.IGF-IR protein expression

The IGF-IR myocardial protein concentration analyzed by Western blot, was higher (60%) in hyperthyroid group than control at the end of 4 weeks treatment with L-thyroxine. Vitamin E treatment decreased (17%) IGF-IR protein expression induced by L-thyroxine (Figure 2D). IGF-IR protein levels were positively correlated with the hypertrophy index and LVEDP values ($r=0.90$, $P<0.05$; $r=0.74$, $P<0.05$, respectively).

4. Discussion

It is well documented the presence of cardiovasculars alterations in clinical and experimental hyperthyroidism, as well as ventricular remodeling phenomenon (cardiac mass and hemodynamics alteration) [24]. We have demonstrated, in a previous work, that ventricular dysfunction in experimental hyperthyroidism is correlated to oxidative damage and antioxidant enzyme changes in protein expression [8]. Thus, we have suggested that oxidative stress is involved in these ventricular alterations. Therefore, treatment with antioxidants would reduce oxidative damage and, thus, improve cardiac function. The present study was designed to test this hypothesis by means a classical antioxidant administration (vitamin E) and to explore one possible mechanism of this physiological answer.

Our results show that chronic hyperthyroid state was successfully achieved, since the elevation in serum T_4, reduction in body weight, and increased heart/body weight ratio were observed in the thyroxine-treated animals. Cardiac myocyte hypertrophy is induced by pathological or physiological stimuli. The pathological hypertrophy is associated with an altered pattern of gene expression, fibrosis, ventricular arrhythmias, and cardiac failure, predicting an increased morbidity and mortality [25]. Gene expression modulation, such as myosin heavy chain and calcium transport/regulatory protein have been associated with hypertrophy [26]. Renin-angiotensin system, that also induce ROS production, has been involved in high cardiac output elevation and cardiac hypertrophy in hyperthyroidism [27]. Cardiac hypertrophy provides intraventricular pressure alteration in the hyperthyroid group. According to our results, an increased LVSP with L-thyroxine administration was seen. The augmented intraventricular pressure is positively correlated with cardiac hypertrophy index in this experimental model. This elevation in cardiac mass is a risk factor for electrical disturbances in the heart in hyperthyroidism [6]. There was also increase in the contractility index ($+dP/dt$), which reflects the hypertrophy impact in the cardiac hemodynamics. Our results have also shown an increased LVEDP, which reflects an augmented residual volume, and a reduced relaxaton index ($-dP/dt$), that, thereby, characterize diastolic dysfunction. Thyroxine treatment provided liver and lung congestion, indicating that these animals were in a congestive heart failure stage. Many mechanisms may mediate the progression from cardiac hypertrophy to hemodynamic failure. It is suggested a main role of ROS in the intracellular signaling of this process [28]. In fact, morphometric and hemodynamic parameters improved when vitamin E was

administered, especially hypertrophy and organs congestion indexes. LVEDP did not show improvement, however, the -d*P*/d*t*, that is a more sensitive measurement, returned to normal levels with vitamin E treatment.

The worth of the present study was that myocardial oxidative stress was increased, concomitant with the development of cardiac hypertrophy and failure, and the treatment with a classical antioxidant, vitamin E, reduced oxidative stress and ventricular dysfunction. Our results show significant increase in myocardial lipid peroxidation (LPO) in hyperthyroid group by means two different methods. It was also shown a positive correlation between oxidative damage and cardiac hypertrophy, confirmed by the use vitamin E that was effective in reducing cardiac mass.

In terms of antioxidant enzymes, no changes were found in CAT activity and concentration in the myocardium of hyperthyroid rats. Myocardial SOD activity and expression were found to be increased in the hyperthyroid animals as compared to controls. However, SOD activity and expression decreased with vitamin E treatment. These results suggest that superoxide radicals concentration is increased in heart tissue, and this ROS probably modulates the regulation of this enzyme [29]. The augmented activity and protein expression of GST found in hyperthyroid and vitamin E groups can reflect the important role of GST enzyme in the detoxification of xenobiotics and, therefore, could be involved in L-thyroxine and vitamin E metabolism. GST activity involves an intense participation of GSH in blocking the peroxidation process, altering cellular redox satus. GSH depletion would lead to intracellular signaling for the protein expression regulation [29]. A total GSH decrease was noticed in this study, and it may reinforce that oxidative stress is taking place in cardiac tissue of

hyperthyroid rats [31]. Myocardial oxidative stress, characterized by GSH depletion, could activate redox-sensitive intracellular signaling pathways. We found a negative correlation between total GSH and cardiac mass ($r=-0.85$, $P<0.05$), suggesting that cardiac hypertrophy development is associated with GSH depletion.

Hyperthyroidism can cause increase in cardiac mass by activating growth factors, such as insulin-like growth factor I (IGF-I) [11]. Our results have shown an increase in IGF-IR protein expression in hyperthyroid group, however vitamin E administration reduced IGF-IR levels. Since vitamin E is considered as a classical antioxidant, this result suggests that oxidative stress participates in the control of IGF-IR expression in hyperthyroidism. We have also demonstrated a negative correlation between total GSH content and IGF-IR protein expression ($r= -0.55$, $P<0.05$). The data suggest that redox-dependent mechanism of IGF-IR protein expression may be through an unbalance in GSH metabolism. GSH depletion could act as a triggering event for cardiac mass increment, since IGF-IR protein levels was strongly correlated with the hypertrophy index values ($r= 0.90$, $P<0.05$). These data indicate a role for oxidative stress in IGF-IR protein expression and myocardial hypertrophic answer in experimental hyperthyroidism.

In summary, chronic treatment with thyroxine induces myocardial hypertrophy and failure by means oxidative stress induction. Thyroxine may activate ROS-sensitive pathways that culminate in the expression of some mediator factors for the myocardial hypertrophy, such as IGF-IR. Vitamin E administration ameliorates the morphometric and hemodynamic impairment induced by thyroxine, exhibiting a beneficial role in the ventricular dysfunction. The overall results point out to a perspective of terapeutical strategy targeting

oxidative stress reduction, to decrease its impact in terms of the progression from cardiac hypertrophy to failure in hyperthyroid patients.

Acknowledgements

This work was supported by CNPq and FAPERGS, Brazilian Research Agencies. We would like to acknowledge Weinmann Clinical Analysis Laboratory for thyroid hormone measurements.

5. References

1. Venditti P, Di Meo S (2006) Thyroid hormones-induced oxidative stress. Cell Mol Life Sci 63: 414-434

2. Klein I, Ojamaa K (2001) Thyroid hormone and cardiovascular system. N Engl J Med 344: 501-509

3. Li T, Danelisen I, Belló-Klein A, Singal PK (2000) Effects of probucol on changes of antioxidant enzymes in adriamycin induced cardiomyopathy in rats. Cardiovasc Res 46: 523-530.

4. Das K, Chainy GB (2001) Modulation of rat liver mitochondrial antioxidant defence system by thyroid hormone. Biochim Biophys Acta 1537: 1-13

5. Esterbauer H, Shaur RJ, Zollner H (1991) Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde, and related aldehydes. Free Rad Biol Med 11: 81-128

6. Donatelli M, Assennato P, Abbadi V, Bucalo M L, Compagno V, Lo Vecchio S, Messina L, RussoV, Schembri A, Torregrossa V, Licata G (2003) Cardiac changes in subclinical and overt hyperthyroid women: retrospective study. Int J Cardiol 90: 159-164

7. Sawyer DB, Siwik DA, Xiao L, Pimentel DR, Singh K, Colucci WS (2002) Role of oxidative stress in myocardial hypertrophy and failure. J Mol Cell Cardiol 34: 379-89

8. Araujo ASR, Ribeiro MFM, Enzveiler A, Schenkel P, Fernandes TRG, Partata WA, Irigoyen MC, Llesuy S, Belló-Klein A (2006) Myocardial antioxidant enzyme activities and concentration and glutathione metabolism in experimental hyperthyroidism. Mol Cell Endocrinol 249: 133-139

9. Das DK, Maulik N, Engelman R M (2004) Redox regulation of angiotensin II signaling in the heart. J Cell Med 8: 144-152

10. McMullen JR, Shioi T, Huang W (2004) The insulin-like growth factor 1 receptor induces physiological heart growth via phosphoinositide 3-kinase (p110α) pathway. J Biol Chem 6: 4782-4793

11. Kuo WW, Chu CY, Wu CH, Lin JA, Hsieh YH, Ueng KC, Lee SD, Hsieh DJ, Hsu HH, Chen LM, Huang CY (2005) Impaired IGF-I signaling of hypertrophic heart in the developmental phase of hypertension in geneticaly hypertensive rats. Cell Biochem Funct 23: 325-331

12. Chitra KC, Mathur PP (2004) Vitamin E prevents nonylphenol-induced oxidative stress in testis of rats. Indian J Exp Biol 42: 220-223

13. Ladenson PW, Kieffer JD, Farwell AP, Ridgway EC (1986) Modulation of myocardial L-triiodthyronine receptors in normal, hypothyroid and hyperthyroid rats. Metabolism 35: 5-12

14. Llesuy SF, Milei J, Molina H, Boveris A, Milei S (1985) Comparison of lipid peroxidation and myocardial damage induced by adriamycin and 4'-epiadrimycin in mice. Tumori 71: 241–249

15. Gonzalez-Flecha B, Llesuy S, Boveris A (1991) Hydroperoxide-initiated chemiluminescence: an assay for oxidative stress in biopsies of liver, heart and muscle. Free Rad Biol Med 10: 41–47

16. Buege JA, Aust SD (1978) Microsomal Lipid Peroxidation. Methods Enzymol 52: 302–309

17. Akerboom T, Sies H (1981) Assay of glutathione disulfide and glutathione mixed disulfides in biological samples. Methods Enzymol 77: 373-382

18. Marklund S (1985) Pyrogallol autooxidation. In Greenwald RA (ed) Handbook of Methods for Oxygen Radical Research CRC, Press Boca Raton, FL:, p 243-247

19. Aebi H (1984) Catalase in vitro. Methods Enzymol 105: 121-126

20. Mannervik B, Gluthemberg C (1981) Glutathione transferase. Methods Enzymol 77: 231-235.

21. Laemmli V (1970) Cleavage of structural proteins during the assembly of the head of bacteriophageT4. Nature 227: 680-685

22. Klein D, Kern RM, Sokol RZ (1995) A method for quantification and correction of proteins after transfer to immobilization membranes. Biochem Mol Biol 36: 1

23. Lowry OH, Rosebrough AL, Farr AL, Randall R (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193: 265–275

24. Dörr M, Wolff B, Robinson DM, John U, Lüdemann J, Meng M, Felix SB, Völzke H (2005) The association of thyroid function with cardiac mass and left ventricular hypertrophy. J Clin Endocrinol. Metabolism 90: 673-677

25. Haider A., Larson M, Benjamin E, Levy D (1998). Increased left ventricular mass and hypertrophy are associated with increased risk for sudden death. J Am Cardiol 32: 1454-1459

26. Kiss E, Jakab G, Kranias EG, Edes I (1994) Thyroid hormone-induced alternations in phospholamban protein expression: regulatory effects on sarcoplasmic reticulum Ca^{+2} transport and myocardial relaxation. Circ Res 75: 245-251.

27. Hu LW, Benvenuti LA, Liberti EA., Carneiro-Ramos MS, Barreto-Chaves, ML (2003) Thyroxine-induced cardiac hyperthrophy: influence of adrenergic nervous system versus renin- angiotensin system on myocyte remodeling. Am J Physiol Regul Integ. Comp Physiol 285: 1473-1480

28. Bianchi P, Pimentel D, Murphy M, Colucci W, Parini A (2005) A new hypertrophy mechanism of serotonin in cardiac myocytes: receptor-independent ROS generation. Faseb J 19: 1-15

29. Das K, Chainy GB (2004) Thyroid hormone influences antioxidant defense system in adult rat brain. Neurochem Rese 29: 1755-1766.

30. Liu H, Colavitti R, Rovira II, Finkel T (2005) Redox-dependent transcriptional regulation. Circ Res 97: 967-974

31. Dincer Y, Akca T; Alademir Z; Ilkova H (2002) Effect of oxidative stress on glutathione pathway in red blood cells from patients with insulin dependent diabetes mellitus. Metabolism 51: 1360-2

Table 1 - Morphometric and hemodynamic parameters after 4-week treatment with thyroxine and/or vitamin E.

Groups	Control	Vitamin E	Hyperthyroid	Hyperthyroid+ Vitamin E
Body weight (g)	232±25	211±15	201±18*	227±19
Heart weight (g)	0.70±0.10	0.65±0.05	0.97±0.10*	0.82±0.05●
Heart/body weight x10^3 (mg/g)	3.0±0.02	3.1±0.02	4.9±0.05*	3.7±0.04●
Lung/body weight x10^3 (mg/g)	0.7±0.10	0.70±0.01	1.20±0.30*	0.80±0.10
Liver/body weight x10^3 (mg/g)	3.3±0.4	3.4±0.5	5.3±0.8*	3.5±0.4●
LVSP (mmHg)	127±11	128±9.8	187±10*	149±27#●
LVEDP (mmHg)	4.5±0.7	5.0±0.8	10±1.3*	9.2±2.1#
+d*P*/d*t*	6321±1714	7331±565	9756±2074*	7500±1479●
-d*P*/d*t*	−5104±1474	−6280±674	−8215±1542*	−5895±1330●

LVSP= left ventricular systolic pressure; LVEDP= left ventricular end diastolic pressure;
+d*P*/d*t*= positive pressure derivative; -d*P*/d*t*= negative pressure derivative.
Values are expressed as mean ± SDM of 10 animals per group.
* significantly different from control (P<0.05).
● significantly different from hyperthyroid (P<0.05).
significantly different from vitamin E (P<0.05).

Table 2 - Antioxidant enzyme activities of superoxide dismutase (SOD), catalase (CAT), glutathione S transferase (GST), and total glutathione (GSH) content in cardiac homogenates from the different groups after 4 week treatment.

Groups	Control	Vitamin E	Hyperthyroid	Hyperthyroid+ Vitamin E
CAT (*pmol/mg prot*)	20.0 ± 2.0	19.0 ± 4.4	18.0 ± 3.0	17.0 ± 2.1
SOD (U/*mg prot*)	6.0 ± 1.6	5.7 ± 0.1	11.0 ± 1.8*	7.0 ± 1.2#●
GST (*nmol/min/mg prot*)	11.0 ± 1.3	14.5 ± 3.5*	17.1 ± 2.1*	26.9 ± 6.5#●
Total GSH (*nmol/mg prot*)	1.23 ± 0.14	2.5 ± 0.5*	0.97 ± 0.15 *	1.12 ± 0.12#●

Values are expressed as mean ± SDM, of 10 animals per group.
* significantly different from control (P<0.05).
● significantly different from hyperthyroid (P<0.05).
significantly different from vitamin E (P<0.05).

Captions to figures:

Figure 1: Myocardial markers of oxidative damage to membrane lipids: chemiluminescence (A), and TBARS (B). Data as mean ± SDM from 10 animals in each group. * significantly different from control (P<0.05). ● significantly different from hyperthyroid (P<0.05). # significantly different from vitamin E (P<0.05).

Figure 2: Western blot analysis in cardiac homogenates using CAT antibody (A), Cu/Zn SOD antibody (B), GST antibody (C), and IGF-IR antibody (D). Data as mean ± SDM from 10 animals in each group (one representative gel of five Western blot experiments, showing two bands for each experimental group). * significantly different from control (P<0.05). ● significantly different from hyperthyroid (P<0.05).

.

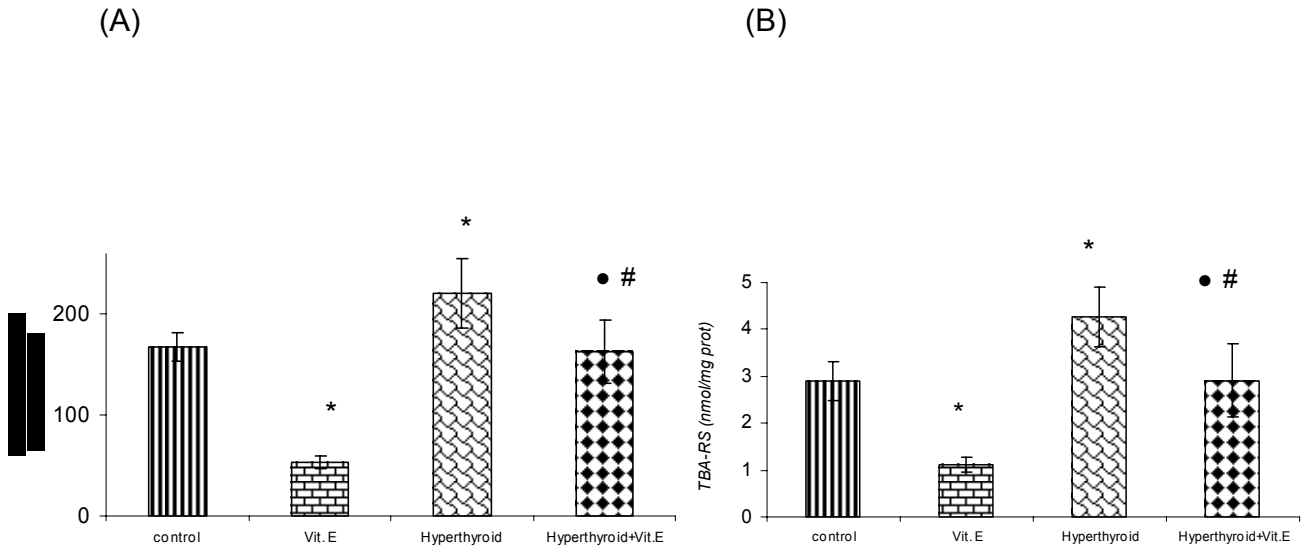

(A)
(B)
200
100
0
control
Vit. E
Hyperthyroid
Hyperthyroid+Vit.E
*
● #
TBA-RS (nmol/mg prot)
5
4
3
2
1
0

Figure 1

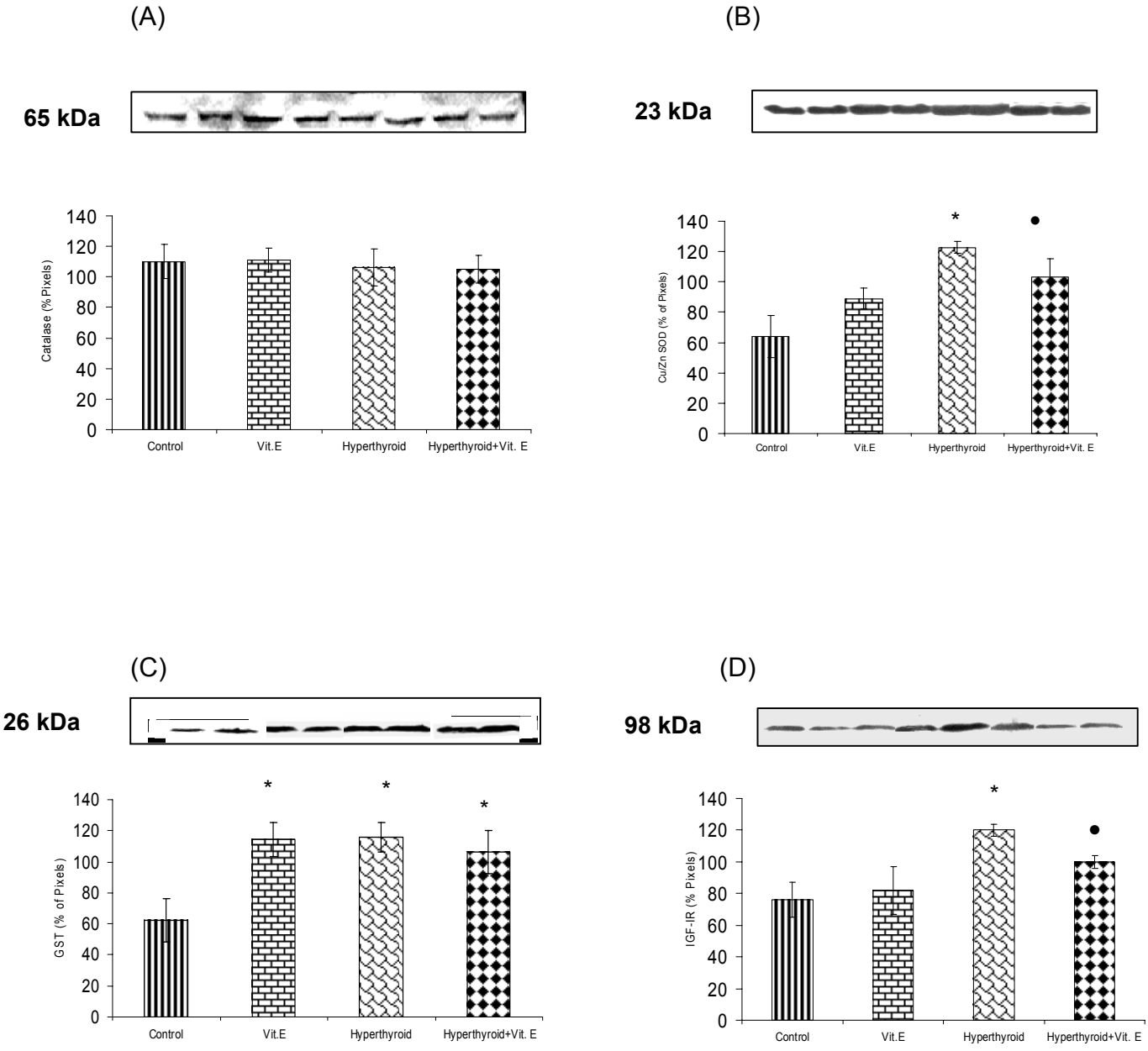

(A)
65 kDa
Catalase (% Pixels)
140
120
100
80
60
40
20
0
Control
Vit.E
Hyperthyroid
Hyperthyroid+Vit. E
(B)
23 kDa
Cu/Zn SOD (% of Pixels)
*
●
Control
Vit.E
Hyperthyroid
Hyperthyroid+Vit. E
(C)
26 kDa
GST (% of Pixels)
*
*
*
(D)
98 kDa
IGF-IR (% Pixels)
*
●

Figure 2

6.3. Artigo 3: *Oxidative stress role in the AKT pathway activation in experimental hyperthyroidism (Journal of Molecular and Cellular Cardiology)*

.

Oxidative stress role in the AKT pathway activation in experimental hyperthyroidism

Araujo, A.S.R.[1]; Schenkel, P.[1]; Enzveiler, A.; T[1].;Fernandes, T.R.G[1]; Partata, W.A.[1]; Irigoyen, M.C.[1]; Llesuy, S.[2]; Ribeiro M.F.M[1]; Belló-Klein, A[1].

[1]*Departamento de Fisiologia, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre - Brasil*

[2] *Cátedra de Química General y Inorgânica, Facultad de Farmácia y Bioquímica, Universidad de Buenos Aires - Argentina*

Corresponding author: Dr. Adriane Belló-Klein

Rua Sarmento Leite, 500.

CEP: 90050-170 - Porto Alegre - RS - Brasil

Email: belklein@ufrgs.br

Telephone: 552151 33163621 Fax: 552151 33163656

Abstract

This study was designed to test the hypothesis that oxidative stress may activate intracellular signaling pathways that culminate with cardiac hypertrophy in the experimental hyperthyroidism. Wistar rats were divided into four groups: control, vitamin E, hyperthyroid, and hyperthyroid+vitamin E. Hyperthyroidism was induced by thyroxine administration (12 mg/L in drinking water during 28 days). Vitamin E treatment was given in the same period by subcutaneous injections (20 mg/kg/day). Morphometric and hemodynamic parameters were evaluated after four weeks. Lipid peroxidation (LPO) by chemiluminescence (CL), protein oxidation by carbonyl assay, glutathione metabolism (GSH/GSSG), total antioxidant capacity (TRAP), hydrogen peroxide (H_2O_2), and nitrates+nitrites were measured in heart homogenates. It was also quantified p-Akt, p-GSK-3β, c-Fos, and c-Jun myocardial protein concentration by Western blot. The contractility index of left ventricle, and cardiac mass were increased by 54% and 60%, respectively, and the relaxation index was decreased by 63% in the hyperthyroid group as compared to control. An increase in CL (32%), carbonyls (41%), H_2O_2 (62%), nitrates+nitrites (218%) was seen in the hyperthyroid group. Hyperthyroidism caused a decrease in GSH (83%) and TRAP (55%). These alterations were reduced by vitamin E administration to hyperthyroid rats. Protein expression of p-Akt/Akt, p-GSK-3β/GSK-3β, c-Fos, and c-Jun were elevated in the hyperthyroid group (by 69%, 37%, 130%, and 33%, respectively), and vitamin E administration promoted a significant reduction in its expression in hyperthyroidism. These results indicate that Akt signaling pathway is activated in

experimental hyperthyroidism by oxidative stress, and H_2O_2 may be a mediator of this intracellular cascade.

***Key words**: thyroxine*, *glutathione, heart failure, vitamin E, hydrogen peroxide, GSK-3β, Akt, c-Fos, c-Jun.*

1. Introduction

Thyroid hormones dysfunction has various effects in cardiovascular system. Hyperthyroidism may cause hemodynamic changes, including increase in myocardial contractility, elevation in cardiac output, a widening of pulse pressure, cardiac arhythmias, such as atrial fibrillation [1]. These changes in electrical and mechanical activity of the heart have been classically attributed to nuclear receptor-mediated regulation of gene transcription [2]. Cardiac hypertrophy in hyperthyroidism may be a consequence of different stimuli, such as pressure overload or neurohormonal activation [3, 4]. Many intracellular pathways and transcription factors have been implicated in the non genomic molecular mechanism of cardiac cell growth. The Akt and GSK-3β signaling pathways have been recently implicated in the regulation of heart growth [5].

The Akt (protein kinase B) family of serine/treonine protein kinases are stimulated classicaly by tyrosine kinase receptors, mediated by the action of phosphatidylinositol 3-kinase (PI3K). Akt pathway induction has been related with cardiac mass increase, through the activation of mTOR-dependent progrowth pathways and suppression of GSK-3β-dependent atrophy programs [6]. The

increase in the basal Akt expression also results in cardiac contractility improvement and hypertrophy [7].

It was recently demonstrated that thyroid hormones may activate the Akt signaling pathway in myocardium and that this process is involved in the cardiac hypertrophy observed in this model [2]. Nevertheless, the mechanisms by which this pathway is activated are still not known. It was demonstrated a positive correlation between oxidative stress and cardiac hypertrophy in experimental hyperthyroidism, indicating that ROS may contribute to the progression to heart failure in this model [8]. Thyroid hormones are involved in the regulation of the basal metabolic state, as well as in the oxidative metabolism. The increased oxygen demand to myocardium, provided by hyperthyroidism, may result in increased generation of ROS [9]. ROS have a high reactivity potential, therefore they are toxic and can lead to oxidative damage to cellular macromolecules such as proteins, lipids, and DNA. Though exist enzymatic and nonenzymatic antioxidant defense systems, when ROS generation exceeds the antioxidant capacity of cells, oxidative stress develops leading to potential tissue damage [10]. ROS are also involved in cellular signaling pathways, not only by modulating a variety of transcription factors (activator protein 1 - AP-1, for example), but also acting as second messengers. Therefore, ROS can play an important role, as molecular mediators, in intracellular cascades, such as those involved in heart growth, inflammatory process, and apoptosis [11].

In this study, we hypothesized that Akt signaling pathway activation, in experimental hyperthyroidism, may be achieved by means oxidative stress and that hydrogen peroxide (H_2O_2) may act as an important mediator of this intracellular cascade.

2. Material and Methods

2.1. Animals

Forty male Wistar rats (250±20 g) were obtained from the Central Animal House of the Universidade Federal do Rio Grande do Sul (UFGRS), Brazil. Animals were housed in plastic cages (four animals each) and received water and pelleted food *ad libitum*. They were maintained under standard laboratory conditions (controlled temperature of 21°C, 12 hours light/dark cycle). Experimental procedures were carried out in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals. The Ethics Committee for Experimental Procedures of UFRGS approved the protocols used in these experiments. Animals were weekly weighed to follow body weight gain, during the experimental protocol (28 days). They were divided into four groups (n=10 per group): a) control, that received water *ad libitum* and daily subcutaneous injections of mineral oil; b) vitamin E, that received subcutaneous injections of vitamin E (20 mg/kg/day in mineral oil) [12]; c) hyperthyroid, which received L-thyroxine (T_4) (12 mg/L in drinking water) [13]; d) hyperthyroid+vitamin E, which received T_4 and vitamin E, at the same conditions as above described.

2.2. Hemodynamic measurements and cardiac hypertrophy development

Cardiac hemodynamics was assessed at the end of the fourth week of thyroxine treatment. In brief, the rats were anesthetized (ketamine 90mg/kg;

xylazine 10mg/kg, i.p.) and the right carotid artery was cannulated with a PE 50 catheter connected to a strain gauge transducer (Narco Biosystem Pulse Transducer RP-155, Houston, Texas, USA) linked to a pressure amplifier (HP 8805C, Hewlett Packard, USA). Pressure readings were taken in a microcomputer equipped with an analogue-to-digital conversion board (CODAS 1kHz sampling frequency, Dataq Instruments, Inc., Akron, Ohio, USA). The catheter was advanced into left ventricle (LV) for recording left ventricular systolic pressure (LVSP, mmHg), left ventricular end diastolic pressure (LVEDP, mmHg), and the positive and negative pressure derivatives ($\pm dP/dt$). Positive derivative of ventricular pressure is considered as the contractility index and the negative derivative is the relaxation index. The cardiac hypertrophy was evaluated by heart weight (in mg) to body weight (in g) ratio.

2.3. Tissue preparation

Four weeks after treatment, rats were decapitated and the hearts were immediatly excised, weighed and homogenized (1.15% w/v KCl and phenyl methyl sulphonyl fluoride PMSF 20 mmol/L) in Ultra-Turrax. The suspension was centrifuged at 1000 g for 10 min at 0-4°C to remove the nuclei and cell debris [14] and supernatants were used for the assay of oxidative stress. Cardiac tissue samples were also rapidly removed and frozen at -80°C, for the evaluation of GSH/GSSG ratio and protein expression and homogeneized as described in sections respectives.

2.4. Thyroid hormones concentration

Blood samples were collected by cardiac punction, and immediately centrifuged at 1000 g for 10 min. After, serum thyroid hormones concentration was measured by chemiluminescence using the Immunolite 2000 kit (Biomedical Technologies, Inc., Strougerton, MA, USA) at Weinmann Clinical Analysis Laboratory.

2.5. Tert-butyl hydroperoxide-initiated chemiluminescence

Chemiluminescence (CL) was measured in a liquid scintillation counter in the out-of-coincidence mode (LKB Rack Beta Liquid Scintillation Spectrometer 1215, LKB – Produkter AB, Sweden). Homogenates were placed in low-potassium vials at a protein concentration of 0.5-1.0 mg/mL in a reaction medium consisting of 120 mmol/L KCl, 30 mmol/L phosphate buffer (pH=7.4). Measurements were started by the addition of 3 mmol/L tert-butyl hydroperoxide and data expressed as counts per second per milligram of homogenates protein (cps/mg protein) [15].

2.6. Carbonyl Assay

Tissue samples were incubated with 2,4 dinitrophenylhydrazine (DNPH 10 mol/L) in 2.5 mol/L HCl solution for 1h at room temperature, in the dark. Samples were vortexed every 15 min. Then, in tube samples was added 20% TCA (w/v) solution, left in ice for 10 min and centrifuged for 5 min at 1000 g, to collect protein precipitates. Another wash was performed with 10% TCA (w/v). The pellet

was washed 3 times with ethanol:ethyl acetate (1:1) (v/v). The final precipitates were dissolved in 6 mol/L guanidine hydrochloride solution, were left for 10 min at 37°C, and read at 360 nm [16].

2.7. Determination of oxidized and reduced glutathione concentration

To determine oxidized and reduced glutathione concentration, tissue was deproteinized with 2 mol/L perchloric acid, centrifuged for 10 min at 1000 g and supernatant was neutralized with 2 mol/L potassium hydroxide. The reaction medium contained 100 mmol/L phosphate buffer (pH 7.2), 2 mmol/L nicotinamide dinucleotide phosphate acid, 0.2 U/mL glutathione reductase, 70 µmol/L 5,5' dithiobis (2-nitrobenzoic acid). To determine reduced glutathione, the supernatant was neutralized with 2 mol/L potassium hydroxide, to react with 70 µmol/L 5,5' dithiobis (2-nitro benzoic acid) and read at 420 nm [17].

2.8. Total antioxidant capacity (TRAP)

TRAP, which indicates total antioxidant capacity present in homogenates, was measured by chemiluminescence using 2,2'-azo-bis(2-amidinopropane) (ABAP, a source of alkyl peroxyl free radicals) and luminol. A mixture consisting of 20 mmol/L ABAP, 40 µmol/L luminol, and 50 mmol/L phosphate buffer (pH = 7.4) was incubated and a steady-state luminescence arose from the free radical-mediated luminol oxidation. This emission was almost completely quenched by the addition of Trolox (hidrosoluble vitamin E), rendering induction times that are linearly related to the free radical scavenger concentration added. A calibration

curve was obtained by using different Trolox concentrations between 0.2 and 1 µmol/L. Addition of homogenates instead of Trolox elicits an induction time related to the initial amount of sample added [18]. Luminescence was measured in a scintillation counter, in the out-of-coincidence mode and the results were expressed in unit of Trolox.

2.9. Determination of hydrogen peroxide

The assay was based in horseradish peroxidase (HRPO)-mediated oxidation of phenol red by hydrogen peroxide, leading to the formation of a compound that absorbs at 610 nm. Ventricle slices were incubated for 30 min. at 37ºC in phosphate buffer 10 mmol/L (NaCl 140 mmol/L and dextrose 5 mmol/L). The supernatants were transferred to tubes with phenol red 0.28 mmol/L and 8.5 U/mL HRPO. After 5 min incubation, NaOH 1 mol/L was added and it was read at 610 nm. The results were expressed in nmoles H_2O_2 / g tissue [19].

2.10. Determination of nitrates (NO_3^-) and nitrites (NO_2^-)

Nitrites were determined using the Griess reagent, in which a chromophore with a strong absorbance at 540 nm is formed by reaction of nitrite with a mixture of naphthyl- elitenediamine (0.1%) and sulphanilamide (1%). The absorbance was measured in a spectrophotometer to give the nitrite concentration. Nitrates were determined as total nitrites (initial nitrite plus nitrite reduced from nitrate) after its reduction using nitrate reductase from *Aspergillus* species in the presence of NADPH. A standard curve was established with a set

of serial dilutions (10^{-8} –10^{-3} mol/L) of sodium nitrite. Results were expressed as mmol/L [20].

.

2.11. Western Blot Analysis

The tissue was homogenized (Tris 20 mmol/L, NaCl 150 mmol/L, EDTA 5 mmol/L, glycerol 10%, phenyl methyl sulphonyl fluoride-PMSF 20 mmol/L, aprotinin 10µL/mL and leupeptin 10µL/mL) in Ultra-Turrax. The suspension was centrifuged at 1000 g for 10 min at 0-4°C to remove the nuclei and cell debris and supernatants were used for the assay. Thirty micrograms of protein were subjected to one dimensional sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) in a discontinuous system using 12% (w/v) separating gel and stacking gel [21]. The protein separated were transferred to nitrocellulose membranes electrophoretically using buffer pH 8.2, containing 20 mmol/L Tris, 150 mmol/L glycine, methanol 20% (V/V) SDS 0.1% (w/v), in a cooled Bio-Rad TransBlot unit. After non-specific protein-binding sites were blocked with 1 h incubation with non-fat milk in Tris-buffer. The membranes were processed for immunodetection using rabbit anti-total Akt polyclonal antibody, rabbit anti-phospho-Akt (ser657) (60 kDa), rabbit anti-total GSK-3β polyclonal antibody (47 kDa), rabbit anti-phospho- GSK-3β (ser9), rabbit anti c-Jun polyclonal antibody, and goat anti c-Fos polyclonal antibody as primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). The bound primary antibodies were detected using rabbit anti-goat or goat anti-rabbit horseradish peroxidase-conjugate secondary antibodies and membranes were revelead for chemiluminescence. The photographs generated were quantitatively analyzed for

the protein levels with an image densitometer (Imagemaster VDS CI, Amersham Biosciences Europe, IT). The molecular weights of the bands were determined by reference to a standard molecular weight marker (RPN 800 rainbow full range Bio-Rad, CA, USA). The results from each membrane were normalized through Ponceau red method [22]. In addition, p-Akt/total Akt ratio and p-GSK-3 β/total GSK-3 β was also performed.

2.12. Determination of Protein Concentration

Protein was measured by the method of Lowry [23], using bovine serum albumin as standard.

2.13. Statistical analysis

Data were expressed as mean $\pm$ SDM and compared using one way ANOVA complemented with Student-Newmann-Keuls. The correlation between two variables was analyzed by Pearson's correlation. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Cardiac Remodelling in Hyperthyroidism

At the end of 4 week post-treatment period, thyroxine serum levels (in ng/mL) were significantly higher in hyperthyroid (24.0±0.7) and

hyperthyroid+vitamin E (19.8±0.8) than control (3.8±0.1) and vitamin E (3.9±0.2). However, no statistical difference was found between the two hyperthyroid groups. It was noticed a body weight loss (in g) (13%) in hyperthyroid (251±18) animals when compared to control (282±25), and vitamin E administration to hyperthyroid rats avoided this change (277±19). Thyroxine has induced cardiac hypertrophy as detected by heart to body weight ratio (mg/g), which was 60% higher in hyperthyroid group (4.9±0.05) than control (3.0±0.02). However, treatment with vitamin E in hyperthyroid rats normalized these values ($P<0.05$). In terms of intraventricular pressure, hyperthyroidism induced an elevation of left ventricular systolic pressure (LVSP) by 44%, as compared to control. Nevertheless, LVSP was reduced (by 20%) in hyperthyroid group that received vitamin E (Figure 1A). Left ventricular end diastolic pressure (LVEDP) increased by 128%, as compared to control, but no significant decrease in LVEDP was observed with vitamin E treatment (Figure 1B). The positive derivative ($+dP/dt$) increased by 54%, and negative derivative ($-dP/dt$) decreased (by 60%) in the hyperthyroid. However, vitamin E administration, to the hyperthyroid animals, decreased $+dP/dt$ by 23%, as well as $-dP/dt$ by 28% (Figure 1 C).

3.2. Oxidative damage, glutathione metabolism, and total antioxidant capacity (TRAP)

Changes in oxidative damage markers and non-enzymatic antioxidant are summarized in Table 1. Hyperthyroidism state caused a significant elevation (32%) in lipid peroxidation products, as indicated by chemiluminescence in cardiac tissue as compared to control ($P<0.05$). L-Thyroxine treatment resulted

also in increased (41%) myocardial oxidative damage to proteins measured through carbonyl assay as compared to control ($P<0.05$). However, hyperthyroid+vitamin E group has shown reduced lipid and protein oxidation by 25% and 29%, respectively, as compared to hyperthyroid. No difference was found between control and hyperthyroid+vitamin E in terms of oxidative damage ($P<0.05$). It was found that hypertrophy index showed a significant positive correlation with lipid peroxidation values ($r=0.73$, $P<0.05$) and carbonyl groups ($r=0.81$, $P<0.05$). It has also been shown that myocardial glutathione metabolism in the hyperthyroid group was changed. The redox status (GSH/GSSG ratio), an oxidative stress indicator, was found to be significantly reduced (83%) in hyperthyroid rats as compared to control ($P<0.05$) (Table 1). There was, however, a restablishment of these values with vitamin E treatment, but GSH/GSSG values were still minor in hyperthyroid+vitamin E than those in the control group. Hyperthyroidism leads to a decrease (by 55%) in TRAP as compared to control group. These values were normalized with vitamin E treatment ($P<0.05$) (Table 1).

3.3. Hydrogen peroxide and nitrates (NO_3^-) and nitrites (NO_2^-)

Hydrogen peroxide levels were increased (62%), in hyperthyroid group, as compared to control, and hyperthyroid+vitamin E decreased (57%) as compared to hyperthyroid ($P<0.05$) (Figure 2A). Total nitrate and nitrites levels have shown an elevation (218%) in hyperthyroid group, but vitamin E administration reduced significantly this increase (56%) when compared to hyperthyroid ($P<0.05$) (Figure 2B).

3.4. p-Akt/Akt and p-GSK-3β/GSK-3β protein expression

L-Thyroxine treatment to rats resulted in elevation (by 69%) in the protein levels of p-Akt/Akt, evaluated by Western blot, as compared to control ($P<0.05$) (Figure 3A). The treatment of hyperthyroid animals with vitamin E reduced p-Akt/Akt protein expression (by 23%) in comparison to hyperthyroid (Figure 3A). Hyperthyroidism has also induced an increase in p-GSK-3β/GSK-3β protein expression by 37% (Figure 3B) as compared to control ($P<0.05$). The hyperthyroid group treated with vitamin E showed a decrease by 30% in p-GSK-3β/GSK-3β levels as compared to hyperthyroid group.

3.5. c-Jun and c-Fos protein expression

c-Jun and c-Fos myocardial protein concentration in hyperthyroid group, analyzed by Western blot, was higher (33% and 130%, respectively) than control at the end of 4 weeks treatment with L-thyroxine (Figure 4A and 4B). This increase in c-Jun and c-Fos protein levels was positively correlated with the hypertrophy index values ($r=0.79$, $P<0.05$ and $r=0.88$, $P<0.05$, respectively). Vitamin E treatment to hyperthyroid rats decreased c-Jun and c-Fos protein expression, by 25% and 43%, respectively (Figure 4A and 4B).

4. Discussion

Our results show that chronic hyperthyroid state was successfully achieved, since the elevation in serum T_4, and increased heart/body weight ratio were observed in the thyroxine-treated animals. According to our results, an increased LVSP with L-thyroxine administration was seen. The augmented intraventricular pressure is positively correlated with cardiac hypertrophy index in this experimental model. There was also an increase in the contractility index ($+dP/dt$), which reflects the hypertrophy impact in the cardiac hemodynamics. Our results also shown an increased LVEDP, which reflects an augmented residual volume, and a reduced relaxation index ($-dP/dt$), that, thereby, characterize diastolic dysfunction. Many mechanisms may mediate the progression from cardiac hypertrophy to hemodynamic failure. It is suggested a main role of ROS in the intracellular signaling of this process [24]. In fact, most of morphometric and hemodynamic parameters improved when vitamin E was administered especially hypertrophy index. LVEDP did not show improvement, however the $-dP/dt$, that is a more sensitive parameter, returned to normal levels with vitamin E treatment.

Left ventricular dysfunction in the hyperthyroid group was associated with increased myocardial oxidative stress. Our results shown significant increase in myocardial lipid peroxidation (LPO) and protein oxidation in hyperthyroid group. This oxidative injury was reduced by vitamin E administration. These results demonstrate a positive correlation between oxidative damage and cardiac hypertrophy, that was confirmed by the use of vitamin E which was effective in reducing hypertrophy. The enhanced oxidative damage in T_4-treated rats is

consistent with increased levels of hydrogen peroxide, which may play a key role in intracellular signaling mechanism in the redox-dependent heart growth development by thyroid hormones [25]. Other important molecule in the oxidative scenary is nitric oxide (NO), which plays a determinant role in cardiac and vascular function [26]. The determination of nitrates ($NO3^-$) and nitrites ($NO2^-$), that are chemical species derived from NO metabolism, demonstrated enhanced levels in hyperthyroid group. These values were reduced by vitamin E treatment, together with partial recovery of cardiac function and hypertrophy.

The results in this work also demonstrated an enhanced consumption of total antioxidants (TRAP) and an unbalance in GSH/GSSG ratio in hyperthyroid group. Myocardial oxidative stress, characterized by GSH depletion, could activate redox-sensitive intracellular signaling pathways. ROS signaling can be divided in two general mechanism of action: alterations in intracellular redox state and oxidative modification of proteins [27]. There is a negative correlation between GSH/GSSG ratio and enhanced cardiac mass ($r=-0.85$, $P<0.05$), suggesting an association beetwen oxidative stress and cardiac hypertrophy development. GSH/GSSG ratio unbalance may also activate transcription factors, such as c-Fos and c-Jun, whose protein expression is increased in the hyperthyroid group. These results are consistent with literature that shows that low GSH concentration may activate AP-1, which results in the heterodimerization of c-Fos and c-Jun proteins, being a transcriptional complex important for genic expression induction of protein involved in cardiac hypertrophy [28]. In addition, a decreased AP-1 expression, caused by vitamin E administration, prevents the development of cardiac hypertrophy through a ROS-dependent pathway [29]. Our result also shown elevation of H_2O_2 concentration

promoved by hyperthyroidism. This increase of H_2O_2 was accompanied by elevation in c-Fos and c-Jun expression, suggesting that this ROS can be realated in signaling intracellular this transcription factors.

Thyroid hormones have also been implicated in Akt signaling pathway of cardiac regulation [2]. Our results demonstrated increased Akt and GSK 3β phosphorylation in the hyperthyroid group, corroborating previous data in literature [2]. In addition, H_2O_2 levels also followed increased of p-Akt. Akt is rapidly activated, overcoat when cells are exposed to stress situations, such as hyperthyroidism [30]. T_4-induced oxidative stress could activate Akt phosphorylation through enhanced H_2O_2 levels [31], that would act as a molecular mediator. We observed that vitamin E attenuates Akt phosphorylation, indicating the role of ROS in the Akt signaling pathway regulation. Vitamin E administration in euthyroid group decreased Akt and GSK 3β phosphorylation, that present also GSH/GSSG ratio high. These vitamin E effect could be resulted of it anitoxidant action, reinforce that ROS can be mediators in signaling intracellular. However literature data implicate a direct molecular action of vitamin E in GSK 3β phosphorylation [32]. These data suggest that H_2O_2 is implicated in the phosphorylation of Akt, activating heart growth in hyperthyroidism.

The main finding of the present study was to demonstrate the outstanding role of ROS as important molecular mediators in the Akt signaling pathway activation for the ventricular remodeling in hyperthyroidism. Our data strongly suggest that oxidative stress play an important role in intracellular signaling pathways activation that culminate with myocardial cell growth exposed to high chronic thyroxine levels, being hydrogen peroxide (H_2O_2) one of the mediators of

these cascades activation. This study provides evidence that Akt signaling can be a redox-dependent pathway in experimental hyperthyroidism.

Acknowledgements

This work was supported by CNPq, CAPES and FAPERGS, Brazilian Research Agencies. We would like to acknowledge Weinmann Clinical Analysis Laboratory for thyroid hormone measurements.

5. References

[1] Klein I, Ojamaa K. Thyroid hormone and cardiovascular system. N Engl J Med 2001; 344: 501-9

[2] Kuzman AJ, Vogelsang KA, Thomas AT, Martin Gerdes A. L-Thyroxine activates Akt signaling in the heart. J Mol Cell Cardiol 2005; 39: 251-58.

[3] Donatelli M, Assennato P, Abbadi V, Bucalo M L, Compagno V, Lo Vecchio S, et al. Cardiac changes in subclinical and overt hyperthyroid women: retrospective study. International J Cardiol 2003; 90: 159-64.

[4] Hu LW, Benvenuti LA, Liberti EA, Carneiro-Ramos MS, Barreto-Chaves M L. Thyroxine-induced cardiac hyperthrophy: influence of adrenergic nervous system versus renin-angiotensin system on myocyte remodeling. Am J Physiol Regul Integr Comp Physiol 2003; 285: 1473-80.

[5] DeBosch B, Treskov I, Lupu TS, Weinheimer C, Kovacs A, Courtois M Muslin AJ. Akt 1 is required for physiological cardiac growth. Circulation 2006; 2097-104

[6] Antos CL, McKinsey TA, Frey N, Kutschke W, McAnaully J, Shelton JM, et al. Activated glycogen synthase-3 beta suppresses cardiac hypertrophy in vivo. Proc Natl Acad Sci USA 2002; 99: 907-12.

[7] Condorelli G, Drusco A, Stassi G, Bellacosa A, Roncarati R, Iaccarino G, et. al. Akt induces enhanced myocardial contractility and cell size in vivo in transgenic mice. Proc Natl Acad Sci USA 2002. 99: 12333-8.

[8] Araujo ASR, Ribeiro MFM, Enzveiler A, Schenkel P, Fernandes TRG, Partata WA, et al. Myocardial antioxidant enzyme activities and concentration and glutathione metabolism in experimental hyperthyroidism. Mol Cell Endocrinol 2006; 249: 133-39.

[9] Venditti P, Di Meo S. Thyroid hormones-induced oxidative stress. Cell Mol Life Sci 2006; 63: 414-34.

[10] Sawyer D B, Siwik D A, Xiao L, Pimentel D R, Singh K, Colucci W S. Role of oxidative stress in myocardial hypertrophy and failure. J Mol Cell Cardiol 2002; 34: 379-89.

[11] Giordano J F. Oxygen, oxidative stress, hypoxia, and heart failure. J Clin Invest 2005; 115: 500-8

[12] Chitra KC, Mathur PP. Vitamin E prevents nonylphenol-induced oxidative stress in testis of rats. Indian J Exp Biol 2004; 42: 220-23.

[13] Ladenson PW, Kieffer JD, Farwell AP, Ridgway EC. Modulation of myocardial L-triiodthyronine receptors in normal, hypothyroid and hyperthyroid rats. Metabolism 1986; 35: 5-12.

[14] Llesuy SF, Milei J, Molina H, Boveris A, Milei S. Comparison of lipid peroxidation and myocardial damage induced by adriamycin and 4'-epiadriamycin in mice. Tumori 1985; 71: 241–49.

[15] Gonzalez-Flecha B, Llesuy S, Boveris A. Hydroperoxide-initiated chemiluminescence: an assay for oxidative stress in biopsies of liver, heart and muscle. Free Rad Biol Med 1991; 10: 41–7.

[16] Reznick AZ, Packer L. Carbonyl assays for determination of oxidatively modified proteins. Meth Enzymol 1994; 233: 357-63.

[17] Akerboom T, Sies H. Assay of glutathione disulfide and glutathione mixed disulfides in biological samples. Meth Enzymol 1981; 77: 373-82.

[18] Evelson P, Travacio M, Repetto M, Escobar J, Llesuy S, Lissi EA. Evaluation of total reactive antioxidant potential (TRAP) of tissue homogenates and their cytosols. Arch. Biochem Biophys 2001; 388: 261-6.

[19] Pick E. Keisari Y. A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. J Immunol Meth 1980; 38: 161-70.

[20] Granger DL, Anstey N.M, Miller WC, Weinberg JB. Measuring nitric oxide production in human clinical studies. Meth Enzymol 1999; 301: 58-61.

[21] Laemmli V. Cleavage of structural proteins during the assembly of the head of bacteriophageT4. Nature 1970; 227: 680-85.

[22] Klein D, Kern RM, Sokol RZ. A method for quantification and correction of proteins after transfer to immobilization membranes. Biochem Mol Biol 1995; 36: 1.

[23] Lowry OH, Rosebrough AL, Farr AL, Randall R. Protein measurement with the folin phenol reagent. J Biol Chem 1951; 193: 265–75.

[24] Bianchi P, Pimentel D, Murphy M, Colucci W, Parini A. A new hypertrophy mechanism of serotonin in cardiac myocytes: receptor-independent ROS generation. FASEB 2005; 9: 1-15.

[25] Liu H, Colavitti R, Rovira II, Finkel T. Redox-dependent transcriptional regulation. Circ Res 2005; 97: 967-74.

[26] Saraiva RM, Hare JM. Nitric oxide signaling in the cardiovascular system: implications for heart failure. Curr Opin Cardiol 2006; 21:221-8.

[27] Thannickal VJ, Day R M, Klinz S J, Bastien MC, Larios JM, Fanburg BL Ras-dependent and-independent regulation of reactive oxygen species by mitogenic growth factors and TGF-beta 1. FASEB 2000; 14: 1741-8.

[28] Cristofanon S, Dicato M, Ghibelli L, Diederich M. Glutathione as mediator of apoptotic cell signaling pathways. Biochem Pharmacol 2006; DOI: 10.1016/j.bcp.2006.03.026.

[29] Li HL, Huang Y, Zhang CN, Liu G, Wei YS, Wang AB, et al. Epigallocathechin-3 gallate inhibits cardiac hypertrophy through blocking reactive oxidative species-dependent and -independent signal pathways. Free Radic Biol Med 2006; 40:1756-75.

[30] Cai H, Li Z., Davis M E, Kanner W, Harrison DG, Dudley SC. Akt-dependent phosphorylation of serine 1179 and mitogen-activated protein kinase kinase/extracellular signal-regulated kinase 1/2 cooperatively mediate activation of the endothelial nitric-oxide synthase by hydrogen peroxide. Mol Pharmacol 2003; 63: 325–31.

[31] Yang B, Oo TN, Rizzo V. Lipid rafts mediate H_2O_2 prosurvival effects in cultured endothelial cells. FASEB J 2006*;* 20: 688–97.

[32] Kempna P, Reiter E, Arock M, Azzi A, Zingg JM. Inhibition of HMC-1 mast cell proliferation by vitamin E: involvement of the protein kinase B pathway.J Biol Chem 2004; 279: 50700-9.

Table 1. Myocardial markers of oxidative damage to membrane lipids (chemiluminescence), proteins (carbonyl groups), total antioxidant capacity (TRAP), and glutathione metabolism (GSH/GSSG).

Group	Control	Vitamin E	Hyperthyroid	Hyperthyroid+ Vitamin E
Chemiluminescence *(cps/mg prot 10^3)*	160±14	60±6*	225±33*	164±31#
Carbonyl groups *(nmol/mg prot)*	2.7 ± 0.1	1.9±0.4*	4.1±0.5 *	2.9±0.5#
GSH/GSSG (*redox status*)	15±3	25±5*#	2.85±0.5 *	8.0±2.0*#
TRAP (*U Trolox/mg prot*)	30±6	40±12*#	13±4 *	32±5.0#

Values are expressed as mean ± SDM of 10 animals per group.
* significantly different from control (P<0.05).
# significantly different from hyperthyroid (P<0.05).

Captions to figures:

Figure 1: Hemodynamic measurements: (A) left ventricular systolic pressure (LVSP); (B) left ventricular end diastolic pressure (LVEDP), and (C) left ventricular performance (in mmHg/sec): positive pressure derivative (+d*P*/d*t*) and negative pressure derivative (-d*P*/d*t*). Data as mean ± SDM from 10 animals in each group. * significantly different from control (P<0.05). # significantly different from hyperthyroid (P<0.05).

Figure 2: (A) Hydrogen peroxide *(nmol/g tissue)*, and (B) Nitrates and nitrites *(nmol/L)* in cardiac tissue. Data as mean ± SDM from 10 animals in each group. * significantly different from control (P<0.05). # significantly different from hyperthyroid (P<0.05).

Figure 3: Western blot analysis in cardiac homogenates using: (A) p-Akt (ser657) (*p-Akt (ser657)/Akt ratio*) ; (B) p-GSK-3β (ser9) (*p-GSK-3β (ser9)/GSK-3β ratio*) (one representative gel of five Western blot experiments, showing two bands for each experimental group). Data as mean ± SDM from 10 animals in each group. * significantly different from control (P<0.05). # significantly different from hyperthyroid (P<0.05).

Figure 4: Western blot analysis in cardiac homogenates using: (A) c-Fos, and (B) c-Jun (one representative gel of five Western blot experiments, showing two bands for each experimental group). Data as mean ± SDM from 10 animals in

each group. * significantly different from control (P<0.05). # significantly different from hyperthyroid (P<0.05).

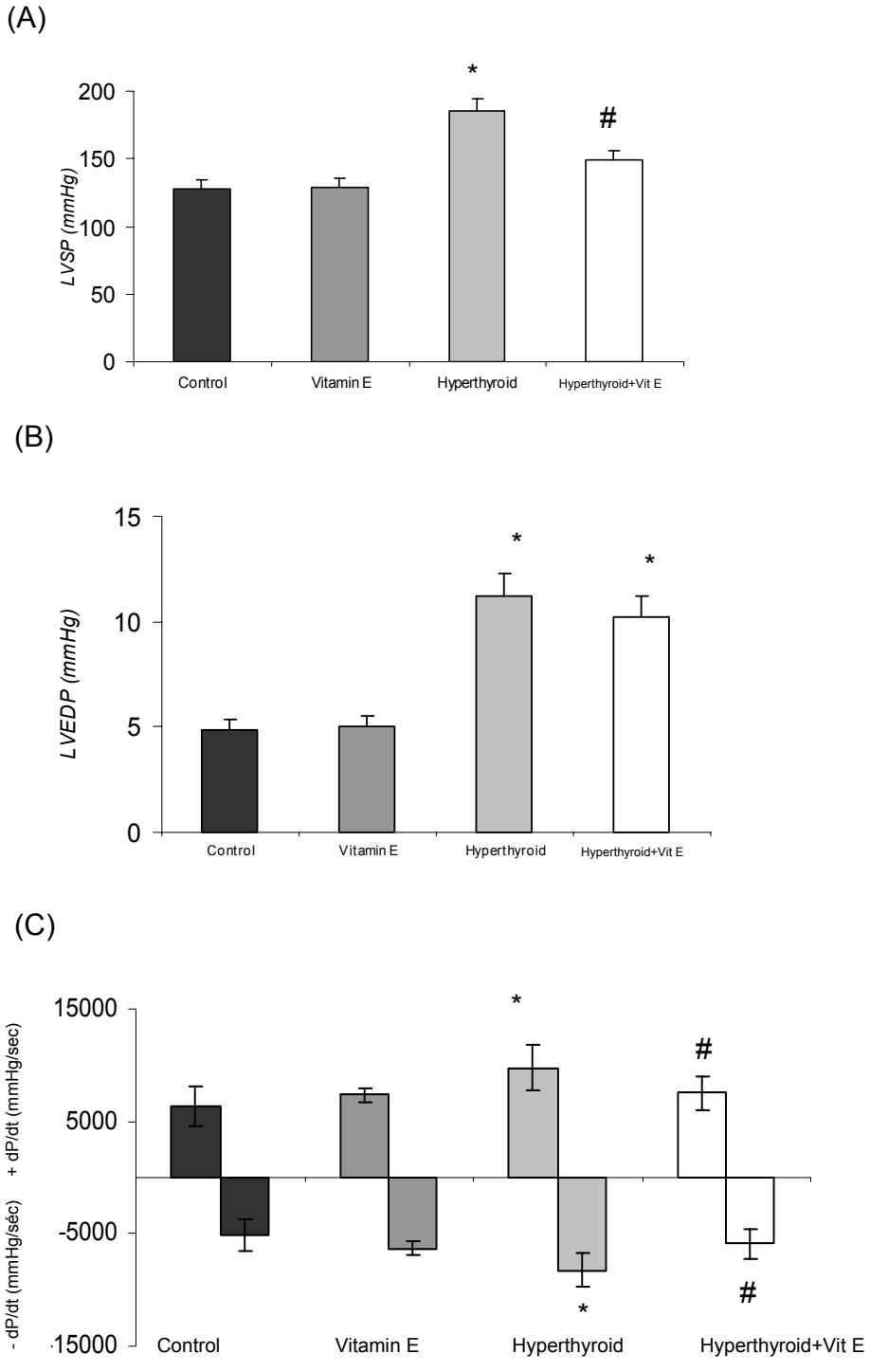

(A)
200
150
100
50
0
LVSP (mmHg)
*
#
Control
Vitamin E
Hyperthyroid
Hyperthyroid+Vit E
(B)
15
10
5
0
LVEDP (mmHg)
*
*
Control
Vitamin E
Hyperthyroid
Hyperthyroid+Vit E
(C)
15000
5000
-5000
-15000
- dP/dt (mmHg/séc)
+ dP/dt (mmHg/sec)
*
#
*
#
Control
Vitamin E
Hyperthyroid
Hyperthyroid+Vit E

Figure 1

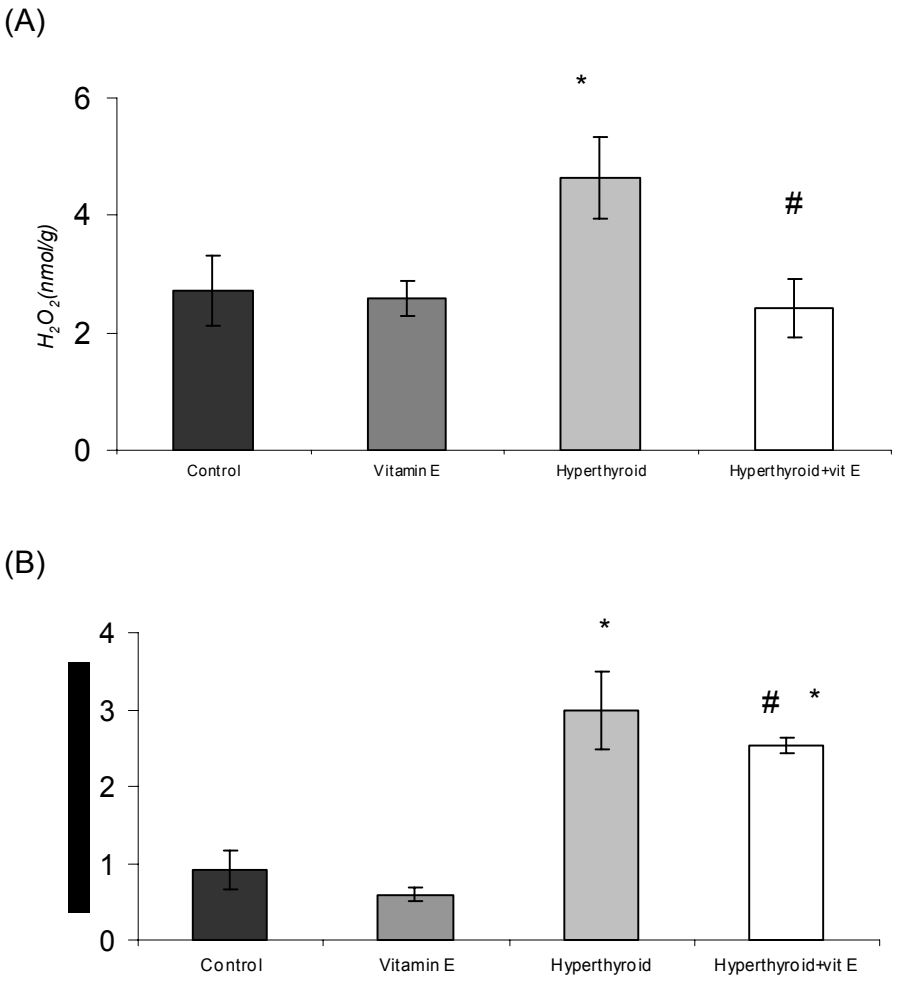
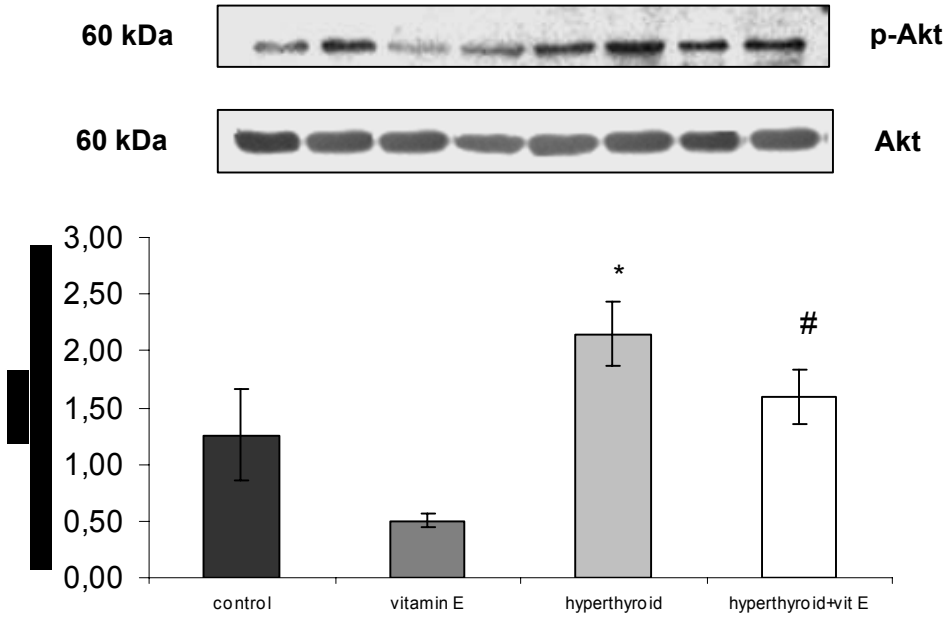

(A)
6
4
2
0
H_2O_2(nmol/g)
*
#
Control
Vitamin E
Hyperthyroid
Hyperthyroid+vit E
(B)
4
3
2
1
0
*
#
*
Control
Vitamin E
Hyperthyroid
Hyperthyroid+vit E

Figure 2

(A)


60 kDa
p-Akt
60 kDa
Akt
3,00
2,50
2,00
1,50
1,00
0,50
0,00
*
#
control
vitamin E
hyperthyroid
hyperthyroid+vit E

(B)

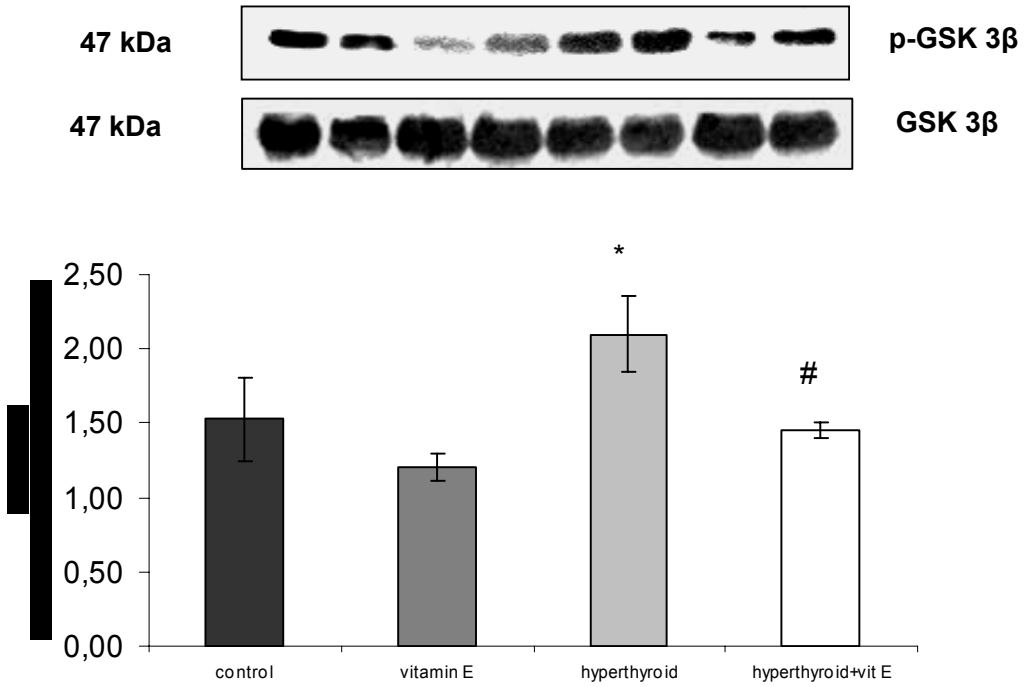
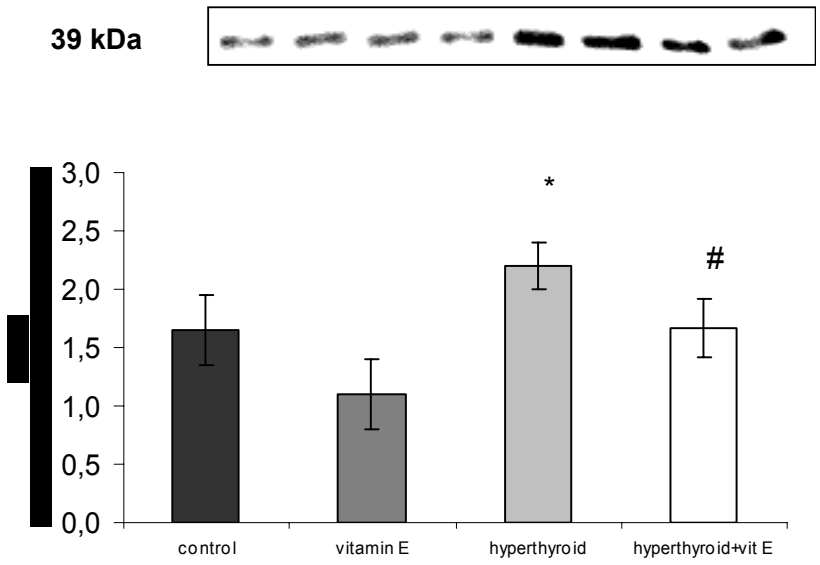

47 kDa
p-GSK 3β
47 kDa
GSK 3β
2,50
2,00
1,50
1,00
0,50
0,00
*
#
control
vitamin E
hyperthyroid
hyperthyroid+vit E

Figure 3

(A)


39 kDa
3,0
2,5
2,0
1,5
1,0
0,5
0,0
*
#
control
vitamin E
hyperthyroid
hyperthyroid+vit E

(B)

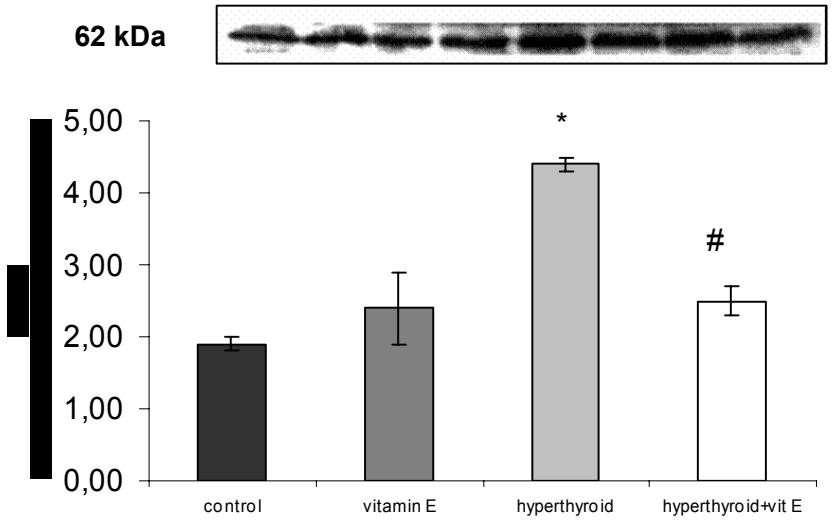

62 kDa
5,00
4,00
3,00
2,00
1,00
0,00
*
#
control
vitamin E
hyperthyroid
hyperthyroid+vit E

Figure 4

7. DISCUSSÃO CONCLUSIVA

O estresse oxidativo está envolvido numa série de processos patológicos e fisiológicos de vários tecidos, exercendo um papel importante no controle da função celular (Dröge, 2002). Dentre os eventos de alteração celular, em que as EAO estão relacionadas, destaca-se o hipertireoidismo. Os hormônios da tireóide podem aumentar o consumo de oxigênio e determinar o aumento da produção de EAO mitocondrial, e por conseqüência, induzir o estresse oxidativo celular pelo desbalanço no equilíbrio entre pró-oxidantes e antioxidantes (Venditti & Di Meo, 2006). No hipertireoidismo, a alteração no estado redox pode levar ao aumento do dano oxidativo nos lipídios e nas proteínas (Venditti & Di Meo, 2006; Araújo, 2006). Ainda como conseqüência das altas concentrações da L-tiroxina, a hipertrofia cardíaca associada às disfunções ventriculares, está bem caracterizada neste modelo (Kuzman *et al*., 2005). Os dados da literatura apresentam, enfaticamente, a relação entre o crescimento da massa ventricular e as EAO. Entretanto, esta correlação é pouco explorada no modelo de hipertireoidismo, deixando sem explicação o mecanismo do desenvolvimento da hipertrofia cardíaca a partir do estresse oxidativo. Por conseguinte, objetivou-se estudar o papel das EAO nas vias de sinalização intracelulares da hipertrofia cardíaca no hipertireoidismo, buscando elucidar possíveis mediadores dessas rotas de sinalização.

No primeiro artigo apresentado, ficou clara a relação do estresse oxidativo com a elevação dos níveis de hormônios tireoidianos, confirmando os dados da literatura (Venditti & Di Meo, 2006). No entanto, houve o inédito

acompanhamento temporal do estresse oxidativo no desenvolvimento do hipertireoidismo. Nesta avaliação, observou-se que, durante as quatro semanas de tratamento com L-tiroxina, o estresse oxidativo se elevou proporcionalmente às alterações hormonais. Entretanto, foram obtidos os valores máximos dos indicadores de dano oxidativo nas duas primeiras semanas de hipertireoidismo. Houve também uma resposta compensatória da atividade das enzimas antioxidantes. Este modelo também foi pioneiro em demonstrar a variação na expressão destas enzimas como resposta ao dano oxidativo causado pelos elevados níveis de L-tiroxina. Um dos indicadores de estresse oxidativo, dado pela razão entre a glutationa reduzida e a glutationa oxidada (GSH/GSSG), indicou que o desbalanço redox iniciava, principalmente, após a segunda semana de tratamento. No âmbito dos aspectos hemodinâmicos e morfométricos cardíacos, verificou-se que o aumento da massa do coração (hipertrofia cardíaca) foi gradual e contínuo durante as quatro semanas de hipertireoidismo, atingindo pico máximo ao final do tratamento com o hormônio tireoidiano. A pressão sistólica ventricular esquerda (PSVE) e a pressão diastólica final do ventrículo esquerdo (PDFVE) também variaram ao longo do tempo, apresentando os maiores níveis ao final do experimento. Cabe salientar que até a segunda semana de tratamento com L-tiroxina a PDFVE não apresentou elevação significativa. Entretanto, a partir da terceira até o final da quarta semana, a PDFVE sofreu um significativo aumento, indicando o estabelecimento da insuficiência cardíaca. Este aumento da PDFVE ocorre concomitante à diminuição da atividade enzimática antioxidante, à depleção da GSH e à piora do estado redox celular. Acompanhando estas variações cardíacas, tem-se a congestão de órgãos, como fígado e pulmões, sugerindo um estágio de

insuficiência cardíaca congestiva. O conjunto destes dados sugere, portanto, que as EAO são precursores das alterações morfométricas e funcionais cardíacas, uma vez que o estresse oxidativo se instalou antes das disfunções ventriculares.

Estes dados abriram a perspectiva de estudar mais profundamente o envolvimento do estresse oxidativo e o mecanismo pelo qual as EAO desencadeiam a hipertrofia cardíaca, juntamente, com as alterações funcionais dela decorrentes. Para tanto, foi necessária a utilização de um antioxidante de ação e eficácia conhecidas, que garantisse o bloqueio da ação dos radicais livres efetivamente. Havia a necessidade de explorar um mecanismo molecular que explicasse a hipertrofia e que confirmasse o envolvimento dos radicais livres. Por conseguinte, foi utilizado um antioxidante clássico, a vitamina E (Devaraj *et al.,* 2005), para verificar a ação dos radicais livres no desenvolvimento da hipertrofia (Kuo *et al.*,2005).

O segundo artigo estudou a relação do estresse oxidativo com o controle da expressão de proteínas do IGF-IR, para averiguar o envolvimento deste fator de crescimento num mecanismo molecular que justificasse o desenvolvimento da hipertrofia cardíaca no hipertireoidismo. O hipertireoidismo, mantido durante quatro semanas, aumentou os níveis de PSVE e de PDFVE, a massa cardíaca e os índices marcadores de estresse oxidativo (lipoperoxidação e a depleção da glutationa total), demonstrando uma correlação positiva entre a hipertrofia e o dano produzido pelos radicais livres. Isto reafirma os dados encontrados no primeiro artigo. Com este modelo bem estabelecido, promoveu-se o tratamento com a vitamina E, clássica molécula antioxidante que impede os efeitos da ação oxidativa dos radicais livres, com o intuito de comprovar a participação do estresse oxidativo no remodelamento cardíaco provocado pelo hipertireoidismo.

O tratamento com a vitamina E diminuiu a lipoperoxidação e a depleção da glutationa total, como já descrito (Pryor *et al*., 2000). Somado a estes resultados, o tratamento com a vitamina E induziu também ao decréscimo da hipertrofia cardíaca nos animais hipertireoideos. Isto indica que o bloqueio da ação dos radicais livres representa uma cárdio-proteção, uma vez que a hipertrofia é fator de risco para alterações elétricas do miocárdio. As derivadas (±dP/dt) também melhoraram, indicando contratilidade e relaxamento melhores devido ao tratamento com vitamina E. A literatura não explicita outro efeito relevante da vitamina E, no sistema cardiovascular, que o seu potencial antioxidante (Tucker and Townsend, 2005). Portanto, estes dados apontam para a participação crítica das EAO no aumento da massa ventricular. Na análise da expressão do IGF-IR, por Western blot, observou-se o aumento da sua expressão nos animais hipertireoideos que regrediu com o tratamento destes animais com a vitamina E. Este acréscimo na quantidade da proteína do IGF-IR apresentou uma correlação positiva com a hipertrofia cardíaca e negativa com o conteúdo da glutationa total nos animais hipertireoideos (Das *et al.,* 2004). A depleção da glutationa pode mudar o estado redox da célula e desencadear uma sinalização intracelular. Esta sinalização poderia aumentar a expressão do IGF-IR e estimular o desenvolvimento da hipertrofia cardíaca. Isto sugere que há uma efetiva participação das EAO e do IGF-IR na hipertrofia e na disfunção cardíaca no hipertireoidismo. Tais alterações foram inibidas pelo tratamento com alfa tocoferol.

Com os dados deste artigo, sugeriu-se o seguinte: a L-tiroxina eleva o consumo de oxigênio e, por conseguinte, aumenta a geração das EAO (Venditti & Di Meo, 2006), o que causaria um desbalanço no estado redox celular e, desta

forma, poderia modular o desenvolvimento da hipertrofia cardíaca, envolvendo o IGF-IR neste processo. No entanto, as vias de sinalização intracelular sensível ao estado redox não estavam bem estabelecidas no nosso modelo de hipertireoidismo experimental, sendo necessário ampliar os objetivos para estudar algumas vias intracelulares de sinalização que estivessem relacionadas com a hipertrofia ventricular desenvolvida pelos hipertireoideos. Portanto, foi avaliada a ativação da via de sinalização da Akt cuja relação com o aumento da massa ventricular, devido aos altos níveis de L-tiroxina, foi recentemente demonstrada (Kuzman *et al.*, 2005).

Os dados da literatura demonstram que a via de sinalização da proteína quinase B (Akt) modula o processo de hipertrofia cardíaca (DeBosch *et al.*, 2006). Entretanto, ainda está obscura a participação dos radicais livres na ativação desta via na promoção do crescimento das células cardíacas no hipertireoidismo. Desta forma, um dos objetivos do terceiro artigo foi esclarecer a participação das EAO na ativação da via Akt de sinalização intracelular que culminaria na hipertrofia do tecido cardíaco. Os resultados obtidos neste experimento indicam um cenário em que os radicais livres parecem modular a fosforilação da Akt, aumentando a sua ativação e a conseqüente inibição da proteína GSK-3β, via que promove a atrofia tecidual. O tratamento com a vitamina E, que previamente tinha prevenido o aumento da ação dos radicais livres e a hipertrofia cardíaca, também inibiu a ativação da via de sinalização da Akt. A fosforilação da Akt e da GSK-3β, juntamente com o estresse oxidativo, também diminuiu com a administração da vitamina E em animais eutireoideos. Isto confirma o papel dos radicais livres como mediadores da sinalização intracelular. No entanto, Kempna *et al*. (2004) relataram o efeito direto da

molécula vitamina E sobre a fosforilação da GSK-3β. Alguns fatores de transcrição também foram analisados neste artigo, sobretudo, o c-Jun e o c-Fos, que são estimulados pelo estresse oxidativo e formam um heterodímero, denominado proteína ativadora -1 (AP-1) (Liu *et al*., 2005). A expressão destes fatores de transcrição estava elevada nos animais hipertireoideos e se correlacionaram positivamente com o estresse oxidativo. No entanto, quando se bloqueou a ação dos radicais livres, pela administração de vitamina E, houve regressão na expressão destes fatores de transcrição, sugerindo que este aumento foi causado pelo desbalanço redox provocado pelo hipertireoidismo (Cai *et al*., 2003). Estabelecida a participação das EAO, faltava ainda determinar quais destas espécies estavam agindo de forma significativa no processo de sinalização intracelular. Desta forma, foram medidos os níveis de peróxido de hidrogênio (H_2O_2) no tecido cardíaco, uma vez que esta é a espécie com maior estabilidade e, portanto, seria a molécula mais provável de atuar como mediadora na sinalização intracelular. Os resultados deste experimento indicaram que as concentrações de H_2O_2 aumentaram nos animais hipertireoideos, e esta elevação se correlacionava não somente com a diminuição da razão GSH/GSSG, mas também com a ativação da via de sinalização da Akt. Estes dados indicam o envolvimento do H_2O_2 na geração do estresse oxidativo, sugerindo também que esta molécula poderia ser um importante mediador no processo de sinalização celular. De fato, esta é uma abordagem mais recente no estudo do estresse oxidativo, gerando uma nova visão sobre as EAO que atuariam como mediadores não apenas do dano oxidativo, mas também da sinalização intracelular que culminaria com a hipertrofia cardíaca.

O conjunto destes resultados aponta para um papel relevante das EAO na hipertrofia cardíaca desenvolvida no hipertireoidismo, tanto pela ação direta do H_2O_2 nas vias moleculares de sinalização que conduzem a célula para o crescimento celular, como pelo estresse oxidativo gerado pelo aumento das concentrações das EAO representado pela depleção da glutationa. O desbalanço na razão GSH/GSSG também pode ativar as vias de comunicação intracelular responsáveis pelo estabelecimento da hipertrofia cardíaca. As alterações hemodinâmicas, decorrentes do aumento da massa ventricular, correlacionaram-se com as variações do estresse oxidativo nos animais tratados com a L -tiroxina. Portanto, o tratamento preventivo dos danos oxidativos pode auxiliar significativamente na melhora da disfunção ventricular apresentada no hipertireoidismo.

8. PERSPECTIVAS

Embora os resultados apontem para uma clara participação das EAO na modulação das vias de sinalização intracelular, outros experimentos seriam necessários para definir mais especificamente o papel dos radicais livres no controle da ativação e expressão das proteínas destas e de outras vias de sinalização envolvidas na hipertrofia cardíaca desenvolvida no hipertireoidismo. Poderiam ser avaliados os seguintes parâmetros:

- a expressão de RNA mensageiro das proteínas Akt, GSK-3β e IGF-IR;
- a expressão de proteína e de RNA mensageiro da ERK 1/2 , ASK e P38;
- a hipertrofia cardíaca através da utilização de métodos mais adequados;
- a disfunção ventricular promovida pela L-tiroxina através de instrumentos mais precisos, como a análise ecocardiográfica;

- as vias de sinalização intracelular em diferentes tempos de hipertireoidismo.
- Medir a concentração do fator de crescimento *insulin like*-I IGF-I.

9. REFERÊNCIAS BIBLIOGRÁFICAS

Aebi H. Catalase in vitro. Methods Enzymol 1984; 105: 121-6.

Antos CL, McKinsey TA, Frey N, Kutschke W, McAnaully J, Shelton JM, et al. Activated glycogen synthase-3 beta suppresses cardiac hypertrophy in viv. Proc Natl Acad Sci USA 2002; 99: 907-12.

Akerboom T, Sies H. Assay of glutathione disulfide and glutathione mixed disulfides in biological samples. Meth Enzymol 1981; 77: 373-82.

Araujo ASR, Ribeiro MFM, Enzveiler A, Schenkel P, Fernandes TRG, Partata WA, et al. Myocardial antioxidant enzyme activities and concentration and glutathione metabolism in experimental hyperthyroidism. Mol Cell Endocrinol 2006; 249: 133-39.

Asahi T, Shimabukuro M, Oshiro Y, Yoshida H, Takasu N. Cilazapril prevents cardiac hypertrophy and postischemic myocardial dysfunction in hyperthyroid rats. Thyroid 2001; 11: 1009-15.

Berenji K, Drazner MH, Rothermel BA, Hill JA. Does load-induced ventricular hypertrophy progress to systolic heart failure? Am J Physiol Heart Circ Physiol 2005; 289: H8-H16.

Bergendi l; Benes l; Duracková Z, Ferencik M. Chemistry, physiology and pathology of free radicals. Life Sci 1999; 65: 1865-1874.

Brady P, Terzic A: Essentials of celular heart failure In: Murphy JG: Mayo clinic cardiol review. 2ªed, Lippincott Williams & Wilkins, Philadelphia, 2000.

Buege JA, Aust SD. Microsomal lipid peroxidation. Methods Enzymol 1978; 52: 302–9.

Cai H, Li Z., Davis M E, Kanner W, Harrison DG, Dudley SC. Akt-dependent phosphorylation of serine 1179 and mitogen-activated protein kinase kinase/extracellular signal-regulated kinase 1/2 cooperatively mediate activation of the endothelial nitric-oxide synthase by hydrogen peroxide. Mol Pharmacol 2003; 63: 325–31.

Cristofanon S, Dicato M, Ghibelli L, Diederich M. Glutathione as mediator of apoptotic cell signaling pathways. Biochem Pharmacol 2006; DOI: 10.1016/j.bcp.2006.03.026.

Condorelli G, Drusco A, Stassi G, Bellacosa A, Roncarati R, Iaccarino G, et. al. Akt induces enhance myocardial contractility and cell size in vivo in transgenic mice. Proc Natl Acad Sci USA 2002; 99: 12333-8.

Das DK, Maulik N, Engelman R M. Redox regulation of angiotensin II signaling in the heart. J Cell Med 2004; 8: 144-52.

Das K, Chainy GB. Modulation of rat liver mitochondrial antioxidant defence system by thyroid hormone. Biochim Biophys Acta 2001; 1537: 1-13.

DeBosch B, Treskov I, Lupu TS, Weinheimer C, Kovacs A, Courtois M Muslin AJ. Akt 1 is required for physiological cardiac grouwth. Circulation 2006; 2097-104.

Devaraj S, Jialal I. Alpha-tocopherol decreases tumor necrosis factor-alpha mRNA and protein from activated human monocytes by inhibition of 5-lipoxygenase. Free Radic Biol Med 2005; 38:1212-20.

Dröge W. Free radicals in the physiological control of cell function. Physiol Rev 2002; 82: 47-95.

El-Helou V, Dupuis J, Proulx C, Drapeau J, Clement R, Gosselin H, Villeneuve L, Manganas L, Calderone A. Resident nestin+ neural-like cells and fibers are detected in normal and damaged rat myocardium. Hypertension 2005; 46: 1219-25.

Evelson P, Travacio M, Repetto M, Escobar J, Llesuy S, Lissi EA. Evaluation of total reactive antioxidant potential (TRAP) of tissue homogenates and their cytosols. Arch. Biochem Biophys 2001; 388: 261-6.

Flohé L; Gunzler WA. Assay of glutathione peroxidase. Methods Enzymol 1984; 105: 14–121.

Frohlich ED, Apstein C, Chobanian Av. The heart in hypertension. N Engl J Med 1992; 327: 998.

Giordano JF. Oxygen, oxidative stress, hypoxia, and heart failure. J Clin Inv 2005; 115: 500-8.

Gonzalez-Flecha B, Llesuy S, Boveris A. Hydroperoxide-initiated chemiluminescence: an assay for oxidative stress in biopsies of liver, heart and muscle. Free Rad Biol Med 1991; 10: 41–7.

Gredilla R, Barja G, Lopez-Torres M. Thyroid hormone-induced oxidative damage on lipids, glutathione and DNA in the mouse heart. Free Radic Res 2001; 35: 417-25.

Guerrero A, Pamplona R, Portero-Otin M, Barja G, Lopez-Torres M. Effect of thyroid status on lipid composition and peroxidation in the mouse liver. Free Rad Biol Med 1999; 26: 73-80.

Granger DL, Anstey N.M, Miller WC, Weinberg JB. Measuring nitric oxide production in human clinical studies. Meth Enzymol 1999; 301: 58-61.

Guyton AC; Hall J.E. Tratado de Fisiologia Médica. 10ª ed. Guanabra Koogan S.A. Rio de Janeiro, 2002.

Habig WH; Pabst MJ; Jakobi WB. Glutathione S-Transferases. J Biol Chem 1974; 249:7130-7139.

Halliwel B. Lipid peroxidation, antioxidants and cardiovascular disease: How should we move forward? Cardiovasc Res 2000; 47: 410-18.

Halliwel B; Gutteridge JCM. Free Radicals in Biology and Medicine**.** 3ª ed. Oxford University Press, 1999.

Heineke J, Molkentin JD. Regulation of cardiac hypertrophy by intracellular signalling pathways. Nat Rev Mol Cell Biol 2006; 7: 589-600.

Henry, J. B. Clinical Diagnosis and Management by Laboratory Methods**.** 19ª ed. W. B. Sanders Company, 1999.

Hu LW, Benvenuti LA, Liberti EA, Barreto-Chaves M L. Myocardial ultrastructure in cardiac hypertrophy induced by thyroid hormone-an acute study in rats. Virchows Arch 2005; 446: 265–269.

Hu LW, Liberti EA, Carneiro-Ramos MS, Barreto-Chaves M L. Thyroxine-induced cardiac hypertrophy: influence of adrenergic nervous system versus renin-

angiotensin system on myocyte remodeling. Am J Physiol Regul Integr Comp Physiol 2003; 285: 1473–80.

Klein D, Kern RM, Sokol RZ. A method for quantification and correction of proteins after transfer to immobilization membranes. Biochem Mol Biol 1995; 36: 1.

Klein I, Ojamaa K. Thyroid hormone and cardiovascular system. N Engl J Med 2001; 344: 501-9.

Kempna P, Reiter E, Arock M, Azzi A, Zingg JM. Inhibition of HMC-1 mast cell proliferation by vitamin E: involvement of the protein kinase B pathway.J Biol Chem 2004; 279: 50700-9.

Kuo WW, Chu CY, Wu CH, Lin JA, Hsieh YH, Ueng KC, Lee SD, Hsieh DJ, Hsu HH, Chen LM, Huang CY. Impaired IGF-I signaling of hypertrophic heart in the developmental phase of hypertension in geneticaly hypertensive rats. Cell Biochem Funct 2005; 23: 325-31.

Kuzman AJ, Vogelsang KA, Thomas AT, Martin Gerdes A. L-Thyroxine activates Akt signaling in the heart. J Mol Cell Cardiol 2005; 39: 251-58.

Ladenson PW, Kieffer JD, Farwell AP, Ridgway EC. Modulation of myocardial L-triiodthyronine receptors in normal, hypothyroid and hyperthyroid rats. Metabolism 1986; 35: 5-12.

Laemmli V. Cleavage of structural proteins during the assembly of the head of bacteriophageT4. Nature 1970; 227: 680-85.

Li HL, Huang Y, Zhang CN, Liu G, Wei YS, Wang AB, et al. Epigallocathechin-3 gallate inhibits cardiac hypertrophy through blocking reactive oxidative species-dependent and -independent signal pathways. Free Radic Biol Med 2006; 40: 1756-75.

Li T, Danelisen I, Belló-Klein A, Singal PK. Effects of probucol on changes of antioxidant enzymes in adriamycin induced cardiomyopathy in rats. Cardiovasc Res 2000; 46: 523-530.

Liu H, Colavitti R, Rovira II, Finkel T. Redox-dependent transcriptional regulation. Circ Res 2005; 97: 967-74.

Llesuy SF, Milei J, Molina H, Boveris A, Milei S. Comparison of lipid peroxidation and myocardial damage induced by adriamycin and 4'-epiadrimycin in mice. Tumori 1985; 71: 241–49.

Lopez-Torres M, Romero M, Barja G. Effect of thyroid hormones on mitochondrial oxygen free radical production and DNA oxidative damage in the rat heart. Mol Cell Endocrinol 2000; 168: 127-34.

Lowry OH, Rosebrough AL, Farr AL, Randall R. Protein measurement with the folin phenol reagent. J Biol Chem 1951; 193: 265–75.

Mannervik B, Gluthemberg C. Glutathione transferase. Methods Enzymol 1981; 77: 231-235.

Marklund S. Pyrogallol autooxidation. In Greenwald RA (ed) Handbook of Methods for Oxygen Radical Research CRC, Press Boca Raton, FL, 1985: 243-247

Niwa K, Inanami O, Yamamori T, Ohta T, Hamasu T, Kuwabara M. Redox regulation of PI3K/Akt and p53 in bovine aortic endothelial cells exposed to hydrogen peroxide. Antioxid Redox Signal 2003;5:713-22.

Pick E. Keisari Y. A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. J Immunol Meth 1980; 38: 161-70.

Pryor WA. Vitamin E and heart disease: basic science to clinical intervention trials. Free Rad Biol Med 2000: 28: 141-64

Reznick AZ, Packer L. Carbonyl assays for determination of oxidatively modified proteins. Meth Enzymol 1994; 233: 357-63.

Robbins, S. L. Et Al. Patologia Estrutural E Funcional-Robbins. 7ª Ed. Rio de Janeiro. Guanabara Koogan S. A., 2005.

Sawyer D B, Siwik D A, Xiao L, Pimentel D R, Singh K, Colucci W S. Role of oxidative stress in myocardial hypertrophy and failure. J Mol Cell Cardiol 2002; 34: 379-89

See F, Thomas W, Way K, Tzanidis A, Kompa A, Lewis D, Itescu S, Krum H. p38 mitogen-activated protein kinase inhibition improves cardiac function and attenuates left ventricular remodeling following myocardial infarction in the rat. J Am Coll Cardiol 2004; 44: 1679-89

Sies H, Cadenas E. Oxidative stress: damage to intact cells and organs. Philos Trans R Soc Lond B Biol Sci 1985; 311: 617-31.

Singal KP, Khaper N, Ferhamand F, Belló-Klein A. Oxidative stressin congestive heart failure. Curr Cardiol Rep 2000; 2: 201-11.

Tsutsui H. Oxidative stress in heart failure: the role of mitochondria. Intern Med 2001; 40: 1177-82.

Tucker JM, Townsend DM. Alpha-tocopherol: roles in prevention and therapy of human disease. Biomed Pharmacother 2005; 59: 380-7.

Venditti P, Di Meo S. Thyroid hormones-induced oxidative stress. Cell Mol Life Sci 2006; 63: 414-34.

Yang B, Oo TN, Rizzo V. Lipid rafts mediate H_2O_2 prosurvival effects in cultured endothelial cells. FASEB J 2006*;* 20: 688–97.

Wakatsuki T, Schlessinger J, Elson EL. The biochemical response of the heart to hypertension and exercise. Trends Biochem Sci. 2004; 29: 609-17.

Wilkins BJ, Dai YS, Bueno OF, Parsons SA, Xu J, Plank DM, Jones F, Kimball TR, Molkentin JD. Calcineurin/NFAT coupling participates in pathological, but not physiological, cardiac hypertrophy. Circ Res 2004; 94: 110-8.

Yu, P.B. Cellular defenses against damage from reactive oxygen species. Physiological Reviews 1994; 74: 139–162