Clinical & experimental studies
- oxidative stress contributes to the development of hypertension in human, animal and in vitro models

induction of oxidative stress by glutathione depletion resulted in severe hypertension

Some experimental models showed
- tissue levels of O₂⁻ or H₂O₂ or both
- direct vasoconstriction
- 

Antioxidant enzymes and effects of Teropol on the development of hypertension induced by nitric oxide inhibition

Presented by Phongrung Chancharoen
25th July 2007

Antioxidant Enzymes and Effects of Teropol on the Development of Hypertension Induced by Nitric Oxide Inhibition

American Journal of Hypertension 2005; 18:871-877

Juan Sari

Hypertension induced by inhibition of NO synthesis

N^ω-nitro-l-arginine methyl ester (L-NAME)

NOS inhibitor

associated with oxidative stress

Supported by: administration of antioxidant flavonoid (quercetin)

protected hypertension development by L-NAME

Introduction

Some experimental models showed

Hypertension induced by inhibition of NO synthesis

- N^ω-nitro-l-arginine methyl ester (L-NAME) NOS inhibitor

associated with oxidative stress

Supported by: administration of antioxidant flavonoid (quercetin)

protected hypertension development by L-NAME

Phagocytic cells

endothelial cells

Expression constitutive inducible constitutive

Calcium-dependent Yes No Yes

Nitric oxide produced picomoles nanomoles picomoles

Isoforms of NOS

<table>
<thead>
<tr>
<th>Tissue</th>
<th>CNS, nitrergic nerves, macrophages</th>
<th>endothelial cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expression</td>
<td>constitutive</td>
<td>inducible</td>
</tr>
<tr>
<td>Calcium-dependent</td>
<td>Yes</td>
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</tr>
<tr>
<td>NO produced</td>
<td>picomoles</td>
<td>nanomoles</td>
</tr>
</tbody>
</table>
**Maintenance of Vascular Homeostasis**

**Normal Endothelium**
- Vasodilation
- Reduced adhesion of platelets and leukocytes
- Anti-inflammatory
- Inhibition of migration and proliferation of smooth muscle cells
- Antioxidant

**Endothelial Dysfunction**
- Vasocconstriction
- Increased adhesion of platelets and leukocytes
- Pro-inflammatory
- Increased migration and proliferation of smooth muscle cells
- Pro-oxidant

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**Role of NO**
- Vasodilator
- Inhibit platelet function
- Monocyte adhesion
- Inhibit VSM migration & proliferation

**Role of SNS & RAS**
- Vasoconstrictor (Ang II & SNS)
- Antinatriuresis
- Blood clotting

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**The Normal Endothelium**
- Healthy blood vessel (thin, elasticity)

**Endothelium Dysfunction**
- Atherosclerosis blood vessel (thick, hardening, loss elasticity)

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**Vasoactive substances produced by the Endothelium**

**Normal Endothelium**
- Vasodilators (EDRF):
  - Nitric oxide (NO)
  - Prostacyclin
  - Endothelin Derived Hyperpolarizing Factor (EDRF)

**Endothelial Dysfunction**
- Vasoconstrictors (EDCF):
  - Angiotensin II (AngII)
  - Superoxide anions (O_2^-)
  - Endothelin-1
  - Prostaglandin H_2
  - Thromboxane A_2

Control vascular tone and blood pressure

**Normal blood pressure & blood flow**

**High blood pressure & reduce blood flow**
**Antioxidant**

A stable, low–molecular-weight superoxide dismutase (SOD) mimetic that is metal independent and cell membrane permeable.

*Mitchell JB et al; 1990*

**Tempol**

(4-hydroxy-2,5,6-tetramethyl piperidinoyl)

- Use to evaluate the role of oxidative stress in pathogenesis of arterial hypertension in experimental models

*Mitchell JB et al; 1990*

**Methods: Animals**

40 males Wistar rats, 250-275 g
Standard rat diet with 0.5% sodium content + tap water ad libitum

**Methods: Experiment**

- Adm/ Tempol in drinking water (18 mg/kg/day)
- Indirect BP from rat’s tail
- Direct BP&HR = 60 min
- Final MAP = 30 min
- Taken blood sample from femoral catheter
- Measured urinary variable every day
- Urine volume
- Creatinine level
- Na+ in urine
- Proteins
- K+ in urine
- Total 8-OHdG

Oxidative stress marker

**Aims of study**

To determine whether HT induced by L-NAME is associated with dysregulation of the main antioxidant enzymes (SOD, CAT, GPX & GR)

To test whether chronic administration of tempol ameliorates L-NAME-induced HT

**Table**

<table>
<thead>
<tr>
<th>Control</th>
<th>L-NAME</th>
<th>Tempol</th>
<th>L-NAME + Tempol</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 10</td>
<td>n = 10</td>
<td>n = 10</td>
<td>n = 10</td>
</tr>
<tr>
<td>(35 mg/kg/day)</td>
<td>(35 mg/kg/day)</td>
<td>(35 mg/kg/day)</td>
<td>(35 mg/kg/day)</td>
</tr>
</tbody>
</table>

**Figure**

- Tempol treatment of hypertensive rats
  - • Ox production in genetic and secondary forms of hypertension
  - Other studies suggested that a major component of the antihypertensive effect of tempol
  - is related to improved NO availability

*Schwamberger G et al; 2006, Meng X et al; 2003, Peng MZ et al; 2001*

**Chart**

- Graph of blood pressure and heart rate over 6 weeks
- Diagram of blood sampling from femoral catheter

**Figure**

- Diagram of the antioxidant process
  - Enzymes and molecules involved in the process
  - NO, SOD, GSH, and other reactive oxygen species

**Figure**

- Flowchart showing the experimental setup and procedures
  - Administration of drugs and monitoring parameters
**Renal cortex**

**Renal medulla**

**Lt. ventricle**

**Rt. ventricle**

**Sampling**

**Morphology study**

**Snap-frozen in liquid nitrogen -70°C**

**Enzymatic analysis**

**Measured Enzymatic activity**

**Clean**

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**Superoxide dismutase (SOD) Activity**

- KCN: selective Cu-Zn SOD inhibitor

- 3 mL of 50 mmol/L K3PO4 buffer (pH 7.8), 1 mmol/L EDTA, 1 mmol/L dithiothreitol

- 3000 rpm, 10 min, 4°C

- Spectrophotometer

Absorbance at 550 nm

**Samples from renal cortex & medulla, Lt. & Rt. Ventricles**

---

**Preparation of tissue homogenate**

- 50 mmol/L K3PO4 buffer (pH 7.4), 1 mmol/L EDTA, 1 mmol/L dithiothreitol

- Polytron homogenizer

- 3000 rpm, 10 min, 4°C

- Determine [protein] by Lowry method

- **Supernatant**

- **Ketone**

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**Catalase (CAT) Activity**

- 3 mL of 50 mmol/L Na-K-PO4 buffer (pH 7.0), 1 mmol/L EDTA, 1 mmol/L dithiothreitol

- Spectrophotometer

Absorbance at 240 nm, 1 min. monitored

**Samples from renal cortex & medulla, Lt. & Rt. Ventricles**

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**Enzymatic Determination**

- **Superoxide dismutase (SOD) Activity**
  - Cu-Zn SOD Activity
  - Mn SOD Activity

- **Catalase (CAT) Activity**

- **Glutathione peroxidase (GPX) Activity**

- **Glutathione reductase (GR) Activity**

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**Glutathione Peroxidase (GPX) Activity**

- 50 mmol/L K3PO4 buffer (pH 7.6), 2 mmol/L EDTA, 1 mmol/L GSH, 1 mmol/L Na2S2O4, 0.2 mmol/L β-NADPH, 1 U/mL GR

- Spectrophotometer

Absorbance at 340 nm

**Samples from renal cortex & medulla, Lt. & Rt. Ventricles**
Samples from renal cortex & medulla, Lt. & Rt. Ventricles

30 mmol/L K3PO4 buffer (pH 7.6), 2 mmol/L NADPH, & 20 mmol/L oxidized glutathione

Absorbance at 340 nm

Spectrophotometer

Plasma & urinary electrolytes, creatinine

Autoanalyzer

[Urine protein]

Bradford method

Urinary excretion of 8-OHdG

ELISA kit

Means ± S.E.M.

Nested design

• Study tail SBP with time
• Bonferroni method

One-way ANOVA

• Other variables measurement
• Newman-Keuls test

CONTROL

TEMPOL

L-NAME

L-NAME + TEMPOL

* p < 0.001 vs CONTROL
+ p < 0.01 vs L-NAME

CONTROL

TEMPOL

L-NAME

L-NAME + TEMPOL

\( \text{SOD activity} + \text{O}_2^- \)

RENAL CORTEX

RENAL MEDULLA

Lt. VENTRICLE

Rt. VENTRICLE

* p < 0.05 vs CONTROL
+ p < 0.05 vs L-NAME
Tempol reduced oxidative stress?

\[ 2 \text{O}_2^- + \text{NADPH} \rightarrow 2\text{O}_2^- + \text{NADP}^- + \text{H}^+ \]  

**Hypothesized**: NO may act as a scavenger of \( \text{O}_2^- \).

In support of this hypothesis, Tempol-treated, NO-deficient rats showed a reduction to normal SOD activity values, probably due to the SOD mimetic activity of Tempol.

**Result: Mn-SOD Activity**

- **Cu-Zn SOD activity**: Cu-Zn SOD found in cytoplasm
- **Mn SOD activity**: Mn SOD activity in renal cortex & medulla as well as in Lt & Rt ventricles
- **L-NAME + Tempol** can reduce Cu-Zn activity in all tissue.

**Result: Catalase(CAT) Activity**

CAT activity: not affected in all tissues. Tempol did not inhibit CAT activity.

**Result: GPX Activity**

GPX activity: affected in renal cortex & medulla. Tempol could inhibit GPX activity.

\[ \text{ONOO}^- \]  

**Rule out a quantitative deficiency of intracellular SOD isoform**

- SOD activity in L-NAME group: compensatory response to NO deficiency.
Result: GR Activity

- GR activity affected in renal cortex & medulla.
- Tempol could inhibit GR activity.

Plasma and Urinary variables

- Protein (mg/100 g/24 h)
- Urinary excretion of 8-OHdG (mg/100 g/24 h)

Morphology of kidney

- Final body weight (g)
- Kidney weight (mg)
- Ratio Kidney weight vs body weight

Morphology of heart

- Left ventricular weight (mg)
- Ratio Left ventricular weight vs body weight
- Ratio Left ventricular weight vs Right ventricular weight

Discussion

In this study, L-NAME group had a significant increase in urinary 8-OHdG, a sensitive and stable biomarker of oxidative stress in vivo.

Hypertension from chronic NO deficiency is associated with increased oxidative stress.

This study showed

- Long-term Tempol administration attenuates development of hypertension in L-NAME hypertensive rats without affecting ventricular hypertrophy or proteinuria as reported in spontaneously hypertensive rats.
- Previous studies showed Normal control animals no response decrease BP to Tempol administration.

This observation: Tempol reduced 8-OHdG in L-NAME rats. L-NAME induced High BP by increase oxidative stress.

Tempol reduced BP in L-NAME rats but not to normal values. Indicating the participation of factors other than oxidative stress in the pathogenesis of L-NAME-induced hypertension.

Antihypertensive effect of Tempol: natriuresis
The mechanisms by which Tempol reduces BP in L-NAME hypertensive rats are unclear. A leftward shift in the renal pressure – natriuresis relationship may offer an explanation. As these rats had the same sodium excretion as L-NAME control rats but with decreased BP, previous study showed Tempol might reduce BP in L-NAME hypertensive rats by MBF & facilitate sodium excretion.

Antihypertensive effect of Tempol: increase NO availability
It has been proposed that NO inactivation is an important mechanism by which oxidative stress contributes to hypertension.

Administration of Tempol or other antioxidants NO availability & hypertension in rats.
Antihypertensive action is blocked after NOS inhibition.

Hence, antihypertensive actions of long term Tempol treatment were attributed to NO availability via oxidative stress.

Antihypertensive effect of Tempol: improve endothelial dysfunction
Antihypertensive effect of Tempol may derive from an improvement in endothelial dysfunction.
Others study proposed that BP reduction produced by chronic tempol treatment of hypertensive rats accompanied by endothelium-dependent vasodilation.
Antihypertensive effect of Tempol in DOCA-salt rats improves EDHF mediated vasodilation induced by ACh.
The activity of endothelium-derived hyperpolarizing factor (EDHF).

Antihypertensive effect of Tempol: NO-independent pathway
However, Tempol-induced BP reduction in NO-deficient hypertensive rats clearly indicates that the antihypertensive effects of Tempol can also be produced by NO-independent pathways.

Prevention of hypertension development by Tempol is independent of NO availability improvements.
Dahl salt-sensitive rats
Rats with ACTH-induced hypertension.

Antihypertensive effect of Tempol: decrease sympathetic activity
Recent studies showed the effect of Tempol on BP, renal sympathetic nerve activity, HR in SHR & DOCA-salt rats to a greater degree than in control rats. These responses are not altered by L-NAME pretreatment. Activation of the SNS by Tempol reduces BP, inhibit the sympathetic activity.

O2 production contributes to Hypertension development via
Activation of the SNS.

Antihypertensive effect of Tempol: not effect on sympathetic function

In this study
- The absence of any HR changes in tempol-treated rats suggests that the effect on sympathetic function did not play a major role in the antihypertensive effect.
- The study design does not allow other actions of tempol on the SNS to be ruled out.

Conclusion

These results indicate that oxidative stress participates in the established phase of L-NAME induced HT.

Tempol ↓HT by mechanisms that do not appear to be related to sympathetic inhibition.

This type of HT was associated with a compensatory upregulation of antioxidant SOD, GPX, & GR activities that was reversed by Tempol administration.

Protein determination

- **LOWRY METHOD**
  - Ranging 0.1-2 mg protein/mL
- **BRADFORD METHOD**
  - Use gel electrophoresis
  - Ranging 0.005-0.2 mg protein/mL

*Thank you for your attention*