Inflammation of different tissues in spontaneously hypertensive rats

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Abstract: The hypertension is one of chronic vascular diseases, which often implicates multiple tissues causing stroke, cardiac hypertrophy, and renal failure. A growing body of evidence suggests that inflammatory mechanisms are important participants in the pathophysiology of hypertension. In this study, the inflammatory status of these tissues (kidney, liver, heart, and brain) in spontaneous hypertensive rats (SHR) was analyzed and its molecular mechanism was explored. The tissues were dissected from SHR and age-matched control Wistar-Kyoto (WKY) rats to investigate the abundance of inflammation-related mediators (IL-1β, TNFα, ICAM-1, iNOS, C/EBPδ and PPARγ). mRNA levels were determined by reverse transcription-polymerase chain reaction and protein expression was evaluated by Western blot. To evaluate the oxidative stress of tissues, carbonyl protein content and total antioxidant capacity of tissues were detected by spectrophotometry and ferric reduction ability power (FRAP) method. The results suggest that: (1) Expressions of inflammation-related mediators (IL-1β, TNFα, ICAM-1, iNOS, C/EBPδ and PPARγ) in SHR were higher compared with those in WKY rats except no evident increase of IL-1β mRNA in liver and brain in SHR. (2) Tissues in SHR contained obviously increased carbonyl protein (nmol/mg protein) compared to that in WKY rats (8.93±1.08 vs 2.27±0.43 for kidney, 2.23±0.23 vs 0.17±0.02 for heart, 13.42±1.10 vs 5.72±1.01 for brain, respectively, P<0.05). However, no evident difference in the amount of carbonyl protein in liver was detected between SHR and WKY rats. (3) Total antioxidant capacities of kidney, liver, heart and brain were markedly lower in SHR than that in WKY rats (P<0.05). Thus, the present data reveal a higher inflammatory status in the important tissues in SHR and indicate that inflammation might play a potential role in pathogenesis of hypertension and secondary organ complications.

Key words: spontaneously hypertensive rat; inflammation; kidney; liver; heart; brain

自发性高血压大鼠多组织炎症状态

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摘要：高血压是一种慢性血管性疾病，易累及肾、肝、心、脑等组织，引起脑卒中和心、肾损害等并发症。本研究对高血压时肾、肝、心、脑等组织的炎症状态进行了观察。实验采用自发性高血压大鼠(spontaneously hypertensive rat, SHR)和正常血压的Wistar-Kyoto (WKY)大鼠，用RT-PCR和Western blot法观察肾、肝、心、脑等组织炎症相关因子IL-1β、TNFα、ICAM-1、iNOS、C/EBPδ和PPARγ的基因表达；紫外分光光度法观察蛋白质羰基化水平和FRAP法检测组织总抗氧化能力。结果显示：(1) SHR组织炎症相关因子表达较对照WKY增强，除IL-1β mRNA在肝和脑的增加不明显外，其余均有显著性差异(P<0.05)；(2) SHR和WKY大鼠肾、心、脑蛋白质羰基化水平(nmol/mg蛋白)分别为8.93±1.08和2.27±0.43、2.23±0.23和0.17±0.02、13.42±1.10和5.72±1.01，SHR明显增加(P<0.05)；而肝脏蛋白质羰基化水平无明显变化；(3) SHR肾、心、脑总抗氧化能力水平显著低于WKY大鼠(P<0.05)。以上结果表明，SHR多个组织(肾、肝、心和脑)均存在炎症因子被诱导和氧化应激反应等明显的炎症状态，提示炎症可能在高血压及其并发症的病理改变中起重要作用。

关键词：自发性高血压大鼠；炎症；肾；肝；心；脑

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A growing body of evidence suggests that inflammatory mechanisms are important participants in the pathophysiology of hypertension. Angiotensin II (Ang II), a potent vasoconstrictor, has been implicated in vascular inflammation of hypertension[1]. Inhibition of Ang II type 1 (AT1) receptors not only normalizes blood pressure but also reduces inflammation in spontaneously hypertensive rats (SHR)[2]. Nuclear factor κB (NF-κB) is activated by Ang II and mediates Ang II-induced inflammatory response in hypertension[3-6]. The vascular wall of SHR has shown an increase in the mRNA expressions of IL-6, IL-1β and TNFα. Similarly, increased expressions of other markers of inflammation, including ICAM, VCAM, monocyte chemotactic protein (MCP-1) and IL-6, have also been reported in hypertensive rats[4-9]. Inflammation plays an important role in triggering fibrosis of vessels, which is an important aspect of extracellular matrix remodeling in hypertension. Remodeling of large and small arteries contributes to the development and complications of hypertension[1].

Although most investigations to date have focused on the vasculature as a key target of inflammatory and oxidative insults, other tissues are also implicated in the pathological process of hypertension. Cardiac hypertrophy and myocardial infarction, stroke, and renal failure are common clinical complications associated with the development and progression of hypertension. However, little data are available in the current literature concerning the inflammatory evaluation of different tissues beyond vasculature in hypertension. The present study aimed at examining the inflammatory status in different tissues in SHR. The extent of inflammation was evaluated by the expressions of a number of pro-inflammatory genes (IL-1β, TNFα, iNOS) and transcription factors, peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding protein β (C/EBPβ) in the kidney, liver, heart and brain of SHR and control Wistar-Kyoto (WKY) rats. Since inflammatory damage is often accompanied by the over production of reactive oxygen species (ROS), tissue levels of protein carbonyls and total antioxidant capacity were also detected as indices of oxidative stress in kidney, liver, heart, and brain.

The present data indicated that increased pro-inflammatory mediators, enhanced protein oxidation, and decreased capacities of total antioxidant reflected a higher inflammatory status in the important tissues in SHR, which might result in the secondary organ damage associated with the disease.

1 MATERIALS AND METHODS

1.1 Animals
Male SHR and WKY rats at the age of 22 weeks (n=16) were purchased from Shanghai Experimental Animal Center of Chinese Academy of Sciences and housed with free access to water and food.

1.2 Materials
TRI reagent was purchased from Sigma-Aldrich (St. Louis, MO). Antibodies for rat iNOS and PPARγ were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). RT-PCR reagents were purchased from BioDev-Tech (China). Primers were synthesized by Shanghai Sangon (China). All the other chemicals were of analytical grade.

1.3 Blood pressure measurement
Systolic blood pressure (SBP) was measured by the tail-cuff method.

1.4 Total RNA isolation and RT-PCR amplification
RNA isolation was performed according to the manufacturer’s instructions. Total RNA (1.5 μg) was subjected to reverse transcription (RT) to synthesize complementary DNA chain. The synthetic heterodimers were amplified by polymerase chain reaction. The sequences of primers for IL-1β were 5'-CCCTCGTGGCCTTGTTGAGC-TCAA-3' and 5'-GGTGCTGATATCCAGTTGGG-3', primers for TNFα were 5'-CCCTCAACTCAGATCATCTTTCGCTAA-3' and 5'-TCTAAGTACTGTGGCCAG-GTTGACCTC-3'; primers for ICAM-1 were 5'-GGGCGCTTGGAGGTGGAT-3' and 5'-GGAGGCGGGGTCTTGTACC-3'; primers for iNOS were 5'-CCAACCGGAGAGGGGACT-3' and 5'-GGAGGCTTGGCCGGCTGAC-3'; primers for PPARγ were 5'-CCACCATGGAGGGACGAACCT-3' and 5'-TPATCTAAATGCTTCTAATGGATG-3'. GAPDH was used as an internal control. The RT-PCR product was applied on 1.2% agarose gel. Band intensity was measured with Gel Image Analysis Software (Tanon, China).

1.5 Protein extraction and Western blot
Frozen tissues in ice-cold lysis buffer (protein inhibitors cocktail in PBS, pH 7.4) were homogenized on ice, microcentrifuged for 15 min at 4 °C and supernatant was
obtained. Proteins (40 mg) were loaded and separated on a 10% SDS-PAGE and transferred onto nitrocellulose membrane. After blocking, the membrane was incubated with anti-iNOS (1:1 000) or anti-PPARγ (1:200) antibody, respectively, for 2 h followed by washing for 10 min three times with PBS-T buffer, then re-incubated with horseradish peroxidase conjugated secondary antibody for 1 h at room temperature. The blot was visualized by chemiluminescence. Protein level was measured by Lowry assay.

1.6 Detection of carbonyl formation with spectrophotometer

Oxidative damage of protein is accompanied by the formation of protein carbonyl groups and it has been used widely as an index of protein oxidation. Protein carbonyls were determined by the spectrophotometric measurement of the formation of 2,4-dinitrophenylhydrazone derivatives ($\varepsilon_{370} = 22 000 \text{ mol}^{-1} \cdot \text{L} \cdot \text{cm}^{-1}$) [7].

1.7 Total antioxidant capacity of tissues by ferric reduction ability power (FRAP) assay

Tissue proteins were reacted with FRAP solution for 10 min at 37 ºC. Absorbance of each sample was measured at 593 nm. Deionized water and Trolox (6-hydroxy-2,5,8-tetramethylchroman-2-carboxylic acid) were used as blank and standard, respectively.

1.8 Statistical analysis

The experimental data were expressed as mean±SD of 6 independent experiments. Results were analyzed by Student’s $t$ test. $P<0.05$ were considered significant.

2 RESULTS

2.1 Expressions of pro-inflammatory factors and nuclear transcription factors in different tissues in SHR and WKY rats

Fig. 1. mRNA expressions of inflammation-related mediators in kidney, liver, heart and brain in SHR and WKY rats. Total RNA was isolated from kidney, liver, heart and brain, then used to detect mRNA levels of pro-inflammatory cytokines (IL-1β, TNFα, ICAM-1 and iNOS), and transcription factors (C/EBPδ and PPARγ) by RT-PCR. GAPDH transcript levels were also determined as an internal standard. The optical density of each band was expressed as the percentage relative to the corresponding GAPDH band (%) in bar graph. A: Bands corresponding to RT-PCR. B: Bar graph of liver. C: Bar graph of heart. D: Bar graph of brain. E: Bar graph of kidney. Values are shown as mean ± SD (n =6). *$P<0.05$ vs corresponding WKY rats.
Expressions of pro-inflammatory cytokines \textit{IL-1\textbeta}, iNOS, \textit{TNF\textalpha} and adhesion molecule ICAM-1 were analyzed. Semiquantitative RT-PCR analysis demonstrated significantly increased expressions of \textit{TNF\textalpha}, ICAM-1 and iNOS in different tissues including kidney, liver, heart and brain in SHR compared to that in WKY rats \((P<0.05, \text{Fig.1})\). Similarly, the significant up-regulation of \textit{IL-1\textbeta} was also detected in kidney and heart in SHR \((P<0.05, \text{Fig.1})\). However, \textit{IL-1\textbeta} in liver and brain in SHR failed to show significant differences compared with that in control WKY rats. Expression of iNOS protein in tissues was detected by Western blot, as shown in Fig.2. It could be detected in kidney, liver, heart and brain in normotensive WKY rats, but at very low level. Hypertension significantly induced iNOS expression in each tissue in SHR \((P<0.05)\).

The expressions of inflammation-related nuclear transcription factors, PPAR\textgamma and C/EBP\textdelta, were also observed. As shown in Fig.1, mRNA levels of PPAR\textgamma (in kidney, liver, heart and brain) and C/EBP\textdelta (in kidney, liver and heart) were noticeably higher in kidney, liver, heart and brain in SHR than that in WKY rats, similar to the alterations of pro-inflammatory factors. However, C/EBP\textdelta in brain failed to demonstrate marked alteration in both strains. PPAR\textgamma protein was identified by using a monoclonal antibody. Compared with values from WKY rats, significant increases in PPAR\textgamma protein expression were detected in tissues in SHR (by 16.97\% for kidney, 22.06\% for liver, 38.49\% for heart, and 11.45\% for brain) \((P<0.05, \text{Fig.2})\).

### 2.2 Determination of carbonyl protein and total antioxidant capacity

In addition to stimulating the expressions of pro-inflammatory factors, inflammation also can cause oxidative stress by activating a number of oxidases, resulting in increased level of ROS. Protein carbonylation is the result of oxidative modification of protein by ROS and has been used widely as an index of protein oxidation. Kidney, heart, and brain in SHR demonstrated significantly higher levels of protein carbonyl compared to that in WKY rats \((P<0.05, \text{Table 1})\). No significant difference was shown in liver between SHR and WKY rats.

Moreover, oxidative stress is usually accompanied by simultaneous decrease in total antioxidant capacities of tissues. As shown in Table 2, total antioxidant capacities of kidney, liver, heart, and brain in SHR were strikingly lower than those in WKY rats.

![Fig. 2. Protein expressions of iNOS and PPAR\textgamma in kidney, liver, heart, and brain in SHR and WKY rats.](image-url)
than that in WKY rats (P<0.05), indicating oxidative stress in important tissues in SHR.

Table 1. Carbonyl protein levels (nmol/mg protein) of kidney, liver, heart and brain in SHR and WKY rats

<table>
<thead>
<tr>
<th>Tissue</th>
<th>WKY</th>
<th>SHR</th>
</tr>
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<tbody>
<tr>
<td>Kidney</td>
<td>2.27±0.43</td>
<td>8.93±1.08*</td>
</tr>
<tr>
<td>Liver</td>
<td>4.99±0.65</td>
<td>5.56±0.56</td>
</tr>
<tr>
<td>Heart</td>
<td>0.17±0.02</td>
<td>2.23±0.23*</td>
</tr>
<tr>
<td>Brain</td>
<td>5.72±1.01</td>
<td>13.42±1.10*</td>
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</tbody>
</table>

*P<0.05 vs WKY, n=6.

Table 2. Total antioxidant capacity (mmol Trolox/mg protein) of kidney, liver, heart and brain in SHR and WKY rats

<table>
<thead>
<tr>
<th>Tissue</th>
<th>WKY</th>
<th>SHR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>10.13±0.33</td>
<td>0.85±0.13*</td>
</tr>
<tr>
<td>Liver</td>
<td>4.33±0.15</td>
<td>1.68±0.66*</td>
</tr>
<tr>
<td>Heart</td>
<td>1.95±0.16</td>
<td>0.47±0.12*</td>
</tr>
<tr>
<td>Brain</td>
<td>5.71±0.23</td>
<td>2.22±0.12*</td>
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*P<0.05 vs WKY, n=6.

3 DISCUSSION

Ang II plays a pivotal role in inflammatory response of hypertension via activation of NF-κB[3-6]. IL-1β, TNFα, ICAM-1 and iNOS are NF-κB-dependent pro-inflammatory factors. The present study showed that the gene expressions of these factors in kidney, liver, heart and brain from SHR were substantially induced, implying the presence of evident inflammatory response in tissues in SHR. Moreover, we also observed the expressions of PPARγ and C/EBPδ nuclear factors associated with inflammation, in tissues in SHR. C/EBPδ transcription is usually suppressed in normal tissues, whereas its marked induction is involved in pathogenic conditions of a given tissue. For example, C/EBPδ expression is at an undetectable or minor level in normal tissues, and rapidly induced by lipopolysaccharide (LPS) and pro-inflammatory cytokines such as IL-1β, IL-6 and TNFα[9]. In hypertension, C/EBPδ is highly expressed in the vascular smooth muscle cells (VSMCs) derived from SHR, but only slightly expressed in the normal artery[10]. In the present study, SHR demonstrated much higher level of C/EBPδ gene expression in kidney, liver, and heart compared to WKY rats, which was in agreement with the previous study and further confirmed the inflammatory conditions in important tissues in SHR.

PPARγ is a ligand-activated transcription factor and plays beneficial roles in inhibiting inflammation and tissue injury. PPARγ activators reduce Ang II-induced ICAM-1, VCAM-1, E-selectin, CRP and iNOS expressions in endothelial cells. The anti-inflammatory effects of PPARγ may be due to inhibition of the NF-κB and AP-1 signaling pathways[11]. Furthermore, PPARγ also regulates blood pressure via increasing NO release from endothelial cells[12] and depressing VSMC proliferation and migration[13,14]. Thus, PPARγ is a potent vascular protector in hypertension. The present study indicated inflammatory response in tissues from SHR, and the anti-inflammatory nuclear factor PPARγ is therefore hypothesized to be reduced in SHR. In contrast with the hypothesis, we observed considerably higher PPARγ expressions in kidney, liver, heart and brain from SHR. Thus, changes (increases) in PPARγ expression might play a compensatory role in the inflammatory status of tissues in SHR.

Inflammatory damage is often accompanied by the over generation of ROS, such as O₂⁻, HO· and H₂O₂, which results in oxidative stress. Increased protein oxidation (protein carbonyl formation) and decreased total antioxidant capacities detected in important tissues in SHR in the present study indicated the presence of oxidative stress in tissues of hypertensive rats. Oxidative stress has detrimental effects on cellular function. Besides attacking cellular proteins, ROS itself is sufficient for the activation of NF-κB and thereby augments the expressions of pro-inflammatory genes driven by NF-κB[9]. Increased O₂⁻ can also react with NO to form peroxynitrite (ONOO⁻), a very unstable and reactive oxidizing species and inactivating vasodilator NO[2]. The reduced bioavailability of NO has been associated with decrease of NO-mediated vascular relaxation and inhibition of polymorphonuclear leukocyte and platelet adhesion and platelet aggregation[2,13].

In summary, the present study evaluated inflammatory status in different tissues in SHR and WKY rats. Our results suggest there are inflammatory and oxidative stress in important tissues of hypertension and inflammatory response in tissues might compromise organ function and contribute to complications of hypertension. Therefore, interfering with the inflammatory mechanisms in hypertension may have synergistic effect with conventional antihypertensive drugs on the prevention and treatment of hypertension and its complications.

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