Mechanical Strain Induces Expression of C-Reactive Protein in Human Blood Vessels

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ABSTRACT

C-reactive protein (CRP) is a powerful independent risk factor for cardiovascular diseases. Elevated mechanical strain on vessels induces the local expression of proinflammatory cytokines. We hypothesized that mechanical strain on vessels may induce local CRP expression. Human saphenous vein and internal mammary artery (IMA) rings were stretched in vitro with a mechanical strength of 1, 3, or 5 g. Reverse transcription-polymerase chain reaction and enzyme-linked immunosorbent assay results showed that mechanical stretching significantly induced CRP mRNA and protein expression in the saphenous vein and IMA rings in a strength-dependent manner reaching a maximum at a mechanical strength of 3 g, but CRP expression returned at strengths of >5 g. In vessels, mechanical strain-induced CRP expression was blocked by two stretch-activated ion channel (SAC) blockers: GdCl3 and streptomycin. Mechanical strain also increased activation of nuclear factor κB (NF-κB), which was detected with a nonradioactive NF-κB p50/p65 EZ-TFA transcription factor assay. Mechanical strain-induced NF-κB activation was blocked by SAC blockers and the NF-κB inhibitor (SN50, H-Ala-Ala-Val-Ala-Leu-Leu-Pro-Ala-Val-Leu-Leu-Ala-Leu-Leu-Pro-Val-Gln-Arg-Lys-Arg-Gln-Lys-Leu-Met-Pro-OH). SN50 also blocked mechanical strain-induced CRP expression in vessels. In conclusion, mechanical strain induces CRP expression in IMAs and saphenous veins by activating the SAC-induced NF-κB pathway.
transduction. SACs activate several intracellular mechanosensitive signaling pathways, including the nuclear factor κB (NF-κB) pathway (Kumar et al., 2003; Amma et al., 2005). NF-κB regulates the expression of many genes involved in inflammatory and acute stress responses (Piva et al., 2006). NF-κB activation was reported in stretched VSMCs and human aneurysmal walls (Lindeman et al., 2008), but reports concerning stretch-induced CRP expression are lacking.

We examined the hypothesis that mechanical strain induces CRP expression via SAC activation of the NF-κB signaling pathway in human saphenous veins and internal mammary arteries (IMAs).

Materials and Methods

Sample Collection. Our study conformed to the ethical principles outlined in the Declaration of Helsinki. The study protocol was approved by the ethics committee of the First Affiliated Hospital to Sun Yat-sen University (Guangzhou, China). Written informed consent was obtained from all study participants.

Saphenous veins and IMAs were collected from 11 male patients with unstable angina pectoris (coronary stenosis >70% as measured by angiography) who had coronary bypass surgery between August and November 2008 at the First Affiliated Hospital. Patients with acute myocardial infarction and other diseases causing increased CRP (e.g., inflammatory disorders, malignancies, infections) were excluded from the study. Saphenous veins or IMAs with atherosclerotic plaques were also excluded.

Preparation of the Vascular Ring. Immediately after removal, tissues were placed in ice-cold Krebs-Henseleit solution containing 118 mM NaCl, 4.76 mM KCl, 1.2 mM NaH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, 1.25 mM CaCl₂, and 11.0 mM glucose. Vessels were dissected, and endothelial cells were removed by gently rotating the vascular section with the tip of a pair of forceps. Each vessel was cut into 3-mm ring segments, which were divided into different groups according to our study protocol. Ring samples were suspended vertically on stainless steel hooks in a tissue chamber containing Krebs-Henseleit solution at 37°C in an atmosphere of 95% O₂ and 5% CO₂.

The mechanical strength generated by the vascular smooth muscle was measured by using a force transducer (JH-2 type; Aerospace Medical Institute, Beijing, China) and recorded with the BL-420 Experimental System of Biological Function (Chengdu TME Technology Company, Chengdu, China). The resting tension was set to 96-well based enzyme-linked immunosorbent assay. During the assay, the capture probe, a double-stranded biotinylated oligonucleotide containing the DNA binding consensus sequence for NF-κB (5'-GGGACCTTCCC-3'), was mixed with the nuclear extract. The active form of NF-κB was contained in the nuclear extract bound to its consensus sequence. The extract/probe/buffer mixture was then directly transferred to a streptavidin-coated plate. The active NF-κB protein was immobilized on the capture probe bound to the streptavidin plate well, and inactive unbound material was washed away. The bound NF-κB transcription factor subunits, p50 and p65, were detected with specific primary antibodies. A highly sensitive horseradish peroxidase (HRP)-conjugated secondary antibody was then used for detection. This provides sensitive colorimetric detection that can be read in a spectrophotometric plate reader at 450 nm. The wild-type consensus oligonucleotide (not biotinylated) was used as a specific competitor for NF-κB binding to monitor the specificity of the assay. A mutated consensus oligonucleotide that had no effect on NF-κB binding was the internal negative control.

Reverse Transcription-Polymerase Chain Reaction for CRP mRNA. Total RNA was isolated from the vascular rings with TRIzol reagent (Invitrogen, Carlsbad, CA). Reverse transcription-polymerase chain reaction (RT-PCR) was done by using the PrimeScript RT-PCR Kit (TaKaRa Biotechnology Company, Dalian, China). Total RNA (1 μg) was reverse-transcribed into cDNA by using oligo(dT) and PrimeScript RTase. Human CRP primers (forward, 5'-TCCTATGCCACCAAGAGAGCAGA-3'; reverse, 5'-AACAGGTCGCTTTGAGCTTACT-3') (Vainas et al., 2003) were designed to amplify a 440-bp fragment from GenBank accession number M11725. Human β-actin primers (forward, 5'-GATTCTATATGGGCGGAGAAT-3'; reverse, 5'-CCATCTTCTTGCTGAGAATG-3') were designed to amplify a 532-bp fragment. PCR was performed under the following conditions: denaturation for 5 min at 94°C, 32 cycles at 94°C for 30 s, 57°C for 30 s, 72°C for 1 min, and elongation at 72°C for 5 min. Amplification fragments were separated on ethidium bromide staining 1.5% agarose gels and were confirmed by sequencing. The resulting bands were imaged with a charge-coupled device (CCD) camera (UVITEC Limited, Cambridge, UK) and analyzed by using the ImageJ 1.37 system from Wayne Rasband (National Institutes of Health, Bethesda, MD). CRP mRNA expression was normalized to the amount of β-actin mRNA in each sample.

Drugs. Acetylsalicylic acid, prostaglandin F₂α, GdCl₃, and sulfated streptomycin were purchased from Sigma-Aldrich (St. Louis, MO). SN50 and SN50M (Calbiochem, San Diego, CA) were purchased from Merek (Darmstadt, Germany).

Statistical Analysis. Data are indicated as mean ± S.D. Differences between groups were evaluated by using analysis of variance (ANOVA) followed by Scheffe’s post hoc test for intergroup comparisons. P < 0.05 was considered significant. Statistical analysis was...
carried out with the Stata 6.0 computer package (Stata Corporation, College Station, TX).

Results

All 11 patients were male with a mean age of 63 ± 7.4 years and a mean body mass index of 23.2 ± 3.1 kg/m² (Table 1). All patients had a diagnosis of unstable angina due to coronary heart disease; 63.6% (7/11) had a history of hypertension, 36.4% (4/11) had a history of hyperlipidemia, and 36.4% (4/11) had a history of diabetes. According to their medical histories, 100% patients were given aspirin and 72.7% had nitroglycerin, but only 27.3% were given statins. Paired IMAs and saphenous veins were isolated from each patient.

Applying mechanical strengths of 1, 3, and 5 g to IMA and saphenous vein rings induces ring length extensions from the basal lengths in a strength-dependent manner, and saphenous vein rings have more ring length extension than IMA rings under the same mechanical strengths (Table 2). Figure 1 shows that application of mechanical strength resulted in significant up-regulation of CRP mRNA and protein expression in arterial and venous rings. In the IMA rings, application of 1, 3, and 5 g induced increases of 3.27 ± 0.90-, 9.67 ± 3.03-, and 2.80 ± 1.04-fold, respectively, in CRP mRNA expression from the basal level (0-g group) (P < 0.05 versus basal level, n = 11; Fig. 1, A1 and A2). In the venous rings, application of 1, 3, and 5 g induced increases of 3.45 ± 0.71-, 10.27 ± 2.80-, and 2.98 ± 0.92-fold, respectively, in CRP mRNA expression from the basal level (P < 0.05 versus basal level, n = 11; Fig. 1, B1 and B2). The level of hsCRP reached a peak on application of a mechanical strength of 3 g, resulting in increases of 7.28 ± 1.84- and 7.60 ± 2.05-fold from basal levels in the arterial rings and venous rings, respectively (P < 0.05 versus 0-, 1-, and 5-g groups).

The role of SAC activation during mechanical stretch-induced CRP expression in the vascular rings was evaluated with two SAC blockers: GdCl₃ and streptomycin. Pretreatment with GdCl₃ (25 μM) or streptomycin (200 μM) completely blocked the increases in CRP mRNA and protein expressions induced by 3 g in arterial and venous rings (P < 0.05 versus 3-g group; P > 0.05 versus 0-g group) (Fig. 2).

The effects of mechanical stretch on NF-κB activation were evaluated by detecting activations of NF-κB p65 and p50 in the nuclear extract from vascular rings stretched by mechanical force. NF-κB p65/p50 activations increased 2.34 ± 0.46/2.20 ± 0.48-, 3.81 ± 0.83/3.58 ± 0.54-, and 1.65 ± 0.46/1.67 ± 0.33-fold from the basal level in arterial rings and increased 2.57 ± 0.62/2.40 ± 0.75-, 4.14 ± 1.70/3.86 ± 1.11-, and 1.78 ± 0.44/1.75 ± 0.46-fold from the basal level in venous rings after being stretched by 1, 3, or 5 g, respectively, for 20 min (P < 0.05 versus basal level; Fig. 3). NF-κB p65 and p50 activations reached peak levels in both types of rings after being stretched by a 3-g mechanical force (P < 0.05 versus 1- and 5-g group; Fig. 3).

We then examined whether SAC was involved in mechanical stretch-induced NF-κB activation. In arterial and venous

### TABLE 1

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Male patients (n = 11)</th>
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<tr>
<td>Age (years)</td>
<td>63 ± 7.4</td>
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<tr>
<td>Body mass index (kg/m²)</td>
<td>23.2 ± 3.1</td>
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<tr>
<td>Coronary risk factors</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td>6 (54.5%)</td>
</tr>
<tr>
<td>Hypertension*</td>
<td>7 (63.6%)</td>
</tr>
<tr>
<td>Blood pressure (mm Hg)</td>
<td>136 ± 18/80 ± 13</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td></td>
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<tr>
<td>Total cholesterol (mM)</td>
<td>4.85 ± 0.87</td>
</tr>
<tr>
<td>Triglycerides (mM)</td>
<td>1.32 ± 0.26</td>
</tr>
<tr>
<td>HDL cholesterol (mM)</td>
<td>1.06 ± 0.17</td>
</tr>
<tr>
<td>LDL cholesterol (mM)</td>
<td>2.42 ± 0.60</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>4 (36.4%)</td>
</tr>
<tr>
<td>Serum glucose (mM)</td>
<td>5.77 ± 1.35</td>
</tr>
<tr>
<td>Medications</td>
<td></td>
</tr>
<tr>
<td>Aspirin</td>
<td>11 (100%)</td>
</tr>
<tr>
<td>Statin</td>
<td>3 (27.3%)</td>
</tr>
<tr>
<td>Nitroglycerin</td>
<td>8 (72.7%)</td>
</tr>
<tr>
<td>Amlodipine</td>
<td>5 (45.5%)</td>
</tr>
<tr>
<td>Insulin</td>
<td>4 (36.4%)</td>
</tr>
</tbody>
</table>

* Number of patients with antihypertensive treatment or blood pressure >140/90 mm Hg.

† Number of patients with cholesterol-lowering treatment or total cholesterol >6.21 mM. HDL, high-density lipoprotein; LDL, low-density lipoprotein.

### TABLE 2

<table>
<thead>
<tr>
<th>Mechanical Strength</th>
<th>Internal Mammary Artery</th>
<th>Saphenous Vein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 11)</td>
<td>(n = 11)</td>
</tr>
<tr>
<td>0 g</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1 g</td>
<td>10.2 ± 2.2*</td>
<td>13.3 ± 2.7*</td>
</tr>
<tr>
<td>3 g</td>
<td>46.1 ± 5.1*</td>
<td>56.3 ± 6.3*</td>
</tr>
<tr>
<td>5 g</td>
<td>68.2 ± 6.4*</td>
<td>79.4 ± 6.1*</td>
</tr>
</tbody>
</table>

* P < 0.05 vs. basal level.

† P < 0.05 vs. the IMA group after application of an equal amount of mechanical strain.

![Fig. 1. A1, representative RT-PCR analysis of CRP mRNA expression in IMA rings after application of mechanical strains of 0, 1, 3, or 5 g for 20 min. A2, mean fold changes from the basal level of hsCRP protein or the basal ratio of CRP mRNA intensity to β-actin mRNA intensity in the arterial rings after application of 0, 1, 3, or 5 g for 20 min. B1, representative RT-PCR analysis of CRP mRNA expression in saphenous vein rings treated with 0, 1, 3, or 5 g for 20 min. B2, mean fold changes from the basal level of hsCRP protein or the basal ratio of CRP mRNA intensity to β-actin mRNA intensity in venous rings after application of 0, 1, 3, or 5 g for 20 min. Lanes 1 to 4 were stretched by 0-, 1-, 3-, and 5-g mechanical strengths, respectively. The basal hsCRP protein level and basal ratio of CRP mRNA intensity to β-actin mRNA intensity were measured in vascular rings that were not treated with mechanical strain (0 g). †, P < 0.05 versus its basal level. †, P < 0.05 versus 3-g group (n = 11 per group).
rings stretched by 3 g, activations of NF-κB p65 and p50 were completely blocked by treatment with streptomyacin (200 μM) or GdCl₃ (25 μM) (Fig. 4). These effects of streptomyacin and GdCl₃ were similar to those of the NF-κB inhibitor SN50 (18 μM) in inhibiting NF-κB activation (P < 0.05 versus 3-g group; P > 0.05, versus 0-g group). The inactive control peptide, SN50M, had no such effect (P > 0.05 versus 3-g group; P < 0.05 versus 0-g group). These results clearly demonstrated that mechanical strain increased NF-κB p65 and p50 activation by activating SAC. To further clarify that the NF-κB signaling pathway was involved in the induction of CRP expression by mechanical stretch, we tested the effect of SN50 on CRP expression in the vascular rings. SN50 (18 μM) completely inhibited CRP mRNA and protein expression induced by 3 g in the IMA rings and saphenous vein rings (P < 0.05 versus 3-g group; P > 0.05 versus 0-g group), but SN50M (18 μM) did not affect CRP mRNA and protein expression (P > 0.05 versus 3-g group; P < 0.05 versus 0-g group) (Fig. 5).

**Discussion**

The primary finding of our study was that mechanical strain increased CRP expression in the saphenous vein and IMAs in a strength-dependent manner, reaching a peak at a mechanical strength of 3 g. CRP expression partially recovered at strengths of >5 g. CRP expression induced by mechanical strain was blocked by the SAC blockers GdCl₃ and streptomyacin. Mechanical strain also increased NF-κB activation; this effect was blocked by SAC blockers and by an NF-κB inhibitor (SN50). SN50 also blocked CRP expression induced by mechanical strain in vessels.

This is the first time that mechanical strain has been shown to induce CRP expression in vessels, although the phenomenon has been suggested indirectly from clinical phenomena. Increased serum CRP was observed in patients with atherosclerosis (Lusic et al., 2006), hypertension (Li et al., 2005), aneurysms (Vainas et al., 2003), and vein grafts in coronary bypass surgery (Parolari et al., 2007) in which mechanical strain increased. More directly, patients with elevated pulse pressure, which indicates significantly increased mechanical strain on vessels, have been shown to have higher serum hsCRP levels (Abramson et al., 2002). The sources of increased CRP in patients with these diseases are controversial. Evidence has shown that CRP is present in atherosclerotic plaques (Wilson et al., 2007), aneurysmal tissues (Vainas et al., 2003), and diseased vein grafts; VSMCs can also express CRP (Jabs et al., 2002).
Increased hsCRP was believed to be derived from the systemic circulation or release by local vessels (Sun et al., 2005; Gulkarov et al., 2006). The mechanism and relationship between increased mechanical strain and increased hsCRP were unclear. Our demonstration of stretch-induced CRP expression provides new insights into the associations between mechanical strain, inflammatory diseases, and vascular diseases. Mechanical strain is a key modulator of the morphology and function of VSMCs and can lead to apoptosis, hypertrophy, and proliferation, which contribute to the development of atherosclerosis, hypertension, and restenosis (Shaw and Xu, 2003). Atherosclerotic lesions occur mainly in areas where vessels experience elevated stretch stress and low shear stress (Zou et al., 1998; Long et al., 2000; Xu, 2000). In their native environment, veins usually do not develop atherosclerosis, but if they are grafted from a low-pressure environment into high-pressure arterial circulation, veins are likely to develop arteriosclerosis (Zou et al., 1998), which is the main reason for late vein graft failure (Motwani and Topol, 1998; Shaw and Xu, 2003). Essential hypertension (Li et al., 2005), aneurysms (Shimizu et al., 2006; Lindeman et al., 2008), and diseased vein grafts (Zou et al., 1998 and 2000), which lead to increased mechanical strain in vessels, are related to inflammation and atherosclerosis. The latter is believed to be an inflammatory disease that involves leukocyte recruitment and production of proinflammatory mediators, such as adhesion molecules, IL-6, and CRP (Libby et al., 2002; Verma et al., 2006). Mechanical strain has been shown to increase production of IL-6 and adhesion molecules in mouse aortas in smooth muscle cells, human vascular endothelial cells, and vein grafts (Nagel et al., 1994; Zou et al., 2000; Zampetaki et al., 2005).

In conclusion, mechanical strain triggers CRP expression in IMAs and saphenous veins. Its mechanism involves the alteration of SAC gating leading to the transformation of the mechanical stimulus into an electrical or biochemical signal, subsequently leading to translocation of NF-κB to the nucleus of VSMCs.
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References


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