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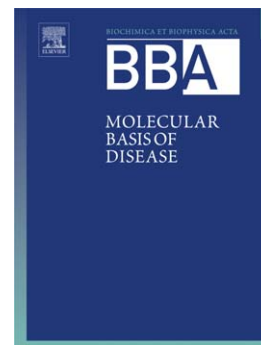
Endothelin-1 and angiotensin II contribute to BNP but not *c-fos* gene expression response to elevated load in isolated mice hearts

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**Endothelin-1 and Angiotensin II Contribute to BNP but not *c-fos* Gene  
Expression Response to Elevated Load in Isolated Mice Hearts**

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### Summary

The early events in the cardiac hypertrophic process induced by hemodynamic load include activation of B-type natriuretic peptide (BNP) and *c-fos* gene expression. However, it is unknown whether stretch acts directly or through local paracrine factors to trigger changes in cardiac gene expression. Herein we studied the involvement of endothelin-1 (ET-1) and angiotensin II (Ang II) in load-induced activation of left ventricular BNP and *c-fos* gene expression using an in vitro stretch model in isolated perfused adult mice hearts. Two-hour stretch induced by increasing coronary flow rate from 2 to 5 ml/min increased the expression of BNP and *c-fos* genes by 1.9- and 1.5 -fold, respectively ( $P < 0.001$  and  $P < 0.05$ ). A mixed ET<sub>A/B</sub> receptor antagonist bosentan attenuated the BNP gene expression response to load by 58% ( $P < 0.005$ ). A similar 53% inhibition was observed with the selective ET<sub>A</sub> receptor blocker BQ-123 ( $P < 0.05$ ). Type 1 Ang II receptor antagonist CV-11974 decreased the activation of BNP gene expression by 50% ( $P < 0.05$ ). In contrast, the activation of *c-fos* gene expression was not inhibited by antagonists of ET<sub>A/B</sub> and AT<sub>1</sub> receptors. Our results show that ET-1 and Ang II play a key role in the induction of BNP, but not *c-fos* gene expression in response to load in intact adult murine hearts.

## 1. Introduction

Pressure overload triggers various adaptive mechanisms in the heart to meet the increased demands. Rapidly after the onset of load, contractile force is increased through the Frank-Starling mechanism followed by a delayed, slow increase in force (slow force response, Anrep effect) [1, 2]. If hemodynamic overload is sustained the myocardium undergoes hypertrophic growth and the increased wall thickness normalizes wall stress and thereby contributes to the maintenance of left ventricular function [3]. The hypertrophic process is characterized by specific changes in cardiac gene expression. Acute hemodynamic overload has been shown to induce a rapid increase in the left ventricular expression of several proto-oncogenes, such as *c-fos* and *c-jun* [4]. Moreover, the expression of B-type natriuretic peptide (BNP) gene takes place with many characteristics of an immediate-early gene. Indeed, mechanical stretch and vasopressin- and phenylephrine-induced hemodynamic overload in the left ventricle have been shown to result in an increase in the BNP gene expression within 1 h [5, 6]. These changes are later followed by reactivation of fetal genes including atrial natriuretic peptide (ANP),  $\beta$ -myosin heavy chain, and skeletal muscle  $\alpha$ -actin [4, 6, 7]. Mechanical stretch of cultured cardiac myocytes causes release of angiotensin II (Ang II) and endothelin-1 (ET-1), which in turn can induce genetic reprogramming and hypertrophic growth. However, it has not yet been established whether stretch acts directly or through local paracrine and autocrine factors liberated in response to hemodynamic load to trigger changes in cardiac gene expression. ET-1 and Ang II have been implicated in stretch-induced early activation of BNP gene expression in cultured rat cardiomyocytes [8] and isolated perfused rat heart preparation [9]. On the other hand, endothelin A/B (ET<sub>A/B</sub>) receptor antagonist bosentan and Ang II type 1 (AT<sub>1</sub>) receptor antagonist losartan failed to inhibit the early activation of left ventricular BNP gene expression in response to pressure overload in vivo in a rat model [10]. Although

genetically engineered mice have been used as disease models at large scale, it is unknown if these paracrine factors are involved in the early phase gene expression responses induced by cardiac overload in the adult, intact murine heart.

In the present study, to examine stretch-induced changes in cardiac gene expression, we applied an in vitro stretch model in isolated perfused adult mice hearts. Elevating aortocoronary perfusion augments coronary perfusion pressure [11] and thereby stretches and thickens the ventricular wall [12]. Immediately, contractile force and oxygen consumption are increased, a response known as the Gregg effect [11]. Moreover, elevated perfusion pressure accelerates protein synthesis in the heart independent of greater cardiac work, development of intraventricular pressure or energy availability [11-14]. In rat heart, elevated perfusion pressure induces the expression of the early response genes, such as *c-fos* and BNP [15]. Increased coronary flow rate stimulates capillary endothelium, which through paracrine mediators is able to regulate cardiomyocyte function [16, 17].

Previously, we have shown that ET-1, but not Ang II, is a key mediator of the contractile response to elevated coronary perfusion in isolated mice hearts [18]. To test the hypothesis that ET-1 or Ang II are involved in the regulation of acute cardiac gene expression response to increased load, ET<sub>A</sub><sup>-</sup>, mixed ET<sub>A/B</sub><sup>-</sup> and AT<sub>1</sub> receptor antagonists were used. We report that the cardiac expression of BNP and *c-fos* was upregulated in mice hearts imposed to elevated coronary perfusion. Furthermore, both local ET-1 and Ang II are involved in the upregulation of BNP but not *c-fos* gene expression in response to increased load in intact mice hearts.

## 2. Materials and methods

### 2.1. *Experimental animals*

Male NMRI mice (10–13 wk of age) obtained from the Experimental Animal Center at the University of Oulu were used for the studies with increased coronary flow rate. The body weight of the mice was 43 g ( $n = 137$ ). The Animal Use and Care Committee of the University of Oulu approved the experimental design.

### 2.2. *Drugs*

The following drugs were used: bosentan, BQ-123, CV-11974 and ET-1. Bosentan was generously supplied by Dr. Martine Clozel, Hoffmann-La Roche (Basel, Switzerland) and Actelion (Allschwil, Switzerland) and CV-11974 by Dr. Hajime Toguchi, Takeda Chemical Industries (Osaka, Japan). BQ-123 and ET-1 were from Phoenix Pharmaceuticals.

### 2.3. *Isolated, perfused mouse heart preparation*

The isolated, perfused mouse heart preparation was similar as previously described [18]. After initialization of retrograde aortic perfusion the hearts were perfused at a constant flow rate of 2 ml/min with a peristaltic pump (model 312, Minipuls 3) for 50 min (equilibration period). Heart rate was maintained steady (400 beats/min) by atrial pacing with a Grass stimulator (8 V, 0.5 ms; model S88, Grass Instruments). Variations in perfusion pressure were measured with a pressure transducer (model MP-15, Micron Instruments) situated on a sidearm of the aortic cannula. All recordings were made with a Grass 7DA polygraph. At the end of the experiment the left and right ventricles were cut separate, weighed, snap frozen in liquid nitrogen and stored in  $-70^{\circ}\text{C}$  until analyzed.

#### 2.4. Experimental design

In the first set of experiments, the level of load required to activate gene expression in mouse hearts was tested by increasing the coronary flow rate after the equilibration period to 4, 5, or 6 ml/min by increasing the rate of the peristaltic pump step by step over a 2-min period. In the experiments characterizing the role of ET-1 and Ang II in responses to elevated flow rate, the 50-min equilibration period was followed by 10 min of pretreatment with vehicle, bosentan (1  $\mu$ M; mixed ET<sub>A/B</sub> receptor antagonist), BQ-123 (100 nM; selective ET<sub>A</sub> receptor antagonist), or CV-11974 (10 nM; Ang II type 1 (AT<sub>1</sub>) receptor antagonist). Thereafter, the infusion was continued and the coronary flow rate was increased from 2 to 5 ml/min for 2 hours. Previous studies have shown that these concentrations of the antagonists effectively block the responses mediated by the respective receptors in isolated hearts [18 - 20].

#### 2.5. Isolation and analysis of cytoplasmic RNA.

RNA was isolated from ventricles by the guanidine thiocyanate-CsCl method [21]. For the RNA Northern blot analyses, 10  $\mu$ g samples of the RNA were transferred to Amersham Hybond N+ nylon membranes. A full-length mouse BNP cDNA probe [22] (a generous gift from Dr. Yoshihiro Ogawa, Kyoto University School of Medicine, Kyoto, Japan), full length rat ANP cDNA probe [23] (a generous gift from Dr Peter L. Davies, Queen's University, Kingston, Canada), cDNA probe made by RT-PCR for rat *c-fos* (nucleotides 231-1280) and cDNA probe complementary to rat 18S ribosomal RNA were labeled with [<sup>32</sup>P]-dCTP with T7 Quick Prime Kit (Pharmacia LKB Biotechnology) and the membranes were hybridized and washed as described previously [15]. The rat probes used showed over 95% homology with respective mouse sequences and a single mRNA species for ANP and *c-fos*, respectively, was identified in Northern blots. Membranes were exposed to

Phosphor screens and scanned with Phosphor Imager (Molecular Dynamics). The hybridization signal was normalized to that of 18S mRNA for each sample. For ET-1 mRNA level analysis, quantitative RT-PCR was used as described [24]. The primer and probe sequences used for prepro-ET-1 analysis were: Forward 5'-ATGGACAAGGAGTGTGTCTACTTCTG-3'; Reverse 5'-GGGACGACGCGCTCG-3'; Probe 5'-Fam-CACCTGGACATCATCTGGGTCAACACTC-Tamra-3'. The sequences for 18S analysis were: Forward 5'-TGGTTGCAAAGCTGAAACTTAAAG-3'; Reverse 5'-AGTCAAATTAAGCCGCAGGC-3'; Probe 5'-Vic-CCTGGTGGTGCCCTTCCGTCA-Tamra-3'

## 2.6. Statistics

Results are expressed as means  $\pm$  SE. Student's *t*-test was used for comparison between two groups. One-way ANOVA followed by post hoc test for least significant differences was used for comparisons with multiple groups. Differences at the 95% level were considered statistically significant.

## 3. Results

### 3.1. Activation of BNP gene expression in response to elevated load in mice hearts

Elevation of coronary flow from the baseline of 2 ml/min to 4, 5 or 6 ml/min for 2 hours resulted in a significant increase in BNP mRNA levels (Fig. 1,  $P < 0.05$  vs. 2 ml/min). The coronary flow rate of 5 ml/min produced the maximal  $1.9 \pm 0.1$ -fold increase in BNP gene expression ( $P < 0.001$ , Fig. 1). Therefore, this flow rate was chosen for further experiments. The changes in cardiac BNP gene expression induced by elevated coronary flow rate were comparable to the changes induced by acute hemodynamic loading *in vivo* [10].

### 3.2. *ET-1 and Ang II mediate the load-induced activation of BNP gene expression.*

A significant attenuation of the BNP gene expression response to elevated load was observed in both ET-1 receptor and AT<sub>1</sub> receptor antagonist treated hearts at two hours. Mixed ET<sub>A/B</sub> receptor antagonist bosentan attenuated the BNP gene expression response to load by 58±11% (Fig. 2,  $P < 0.005$ ), while AT<sub>1</sub> antagonist CV-11974 inhibited the induction of BNP gene expression by 50±10% ( $P < 0.05$ ). Moreover, corresponding (53±10%) attenuation of the load-induced BNP response was seen with the selective ET<sub>A</sub> receptor blocker BQ-123 ( $P < 0.05$ ). Combined administration of bosentan and CV-11974 resulted in 65±7 % smaller BNP response to load compared to vehicle infusion (Fig. 2,  $P < 0.01$ ). However, even the combination was not able to completely prevent the activation of BNP gene expression in response to elevated coronary perfusion ( $P < 0.05$  vs vehicle treated hearts perfused with 2ml/min). Furthermore, there was no statistically significant difference in BNP mRNA levels between the bosentan plus CV-11974 treated hearts compared to the hearts treated with either of these agents alone (Fig. 2). Similarly to previously reported results [10], ET<sub>A/B</sub>, ET<sub>A</sub>, or AT<sub>1</sub> receptor antagonists had no effects on left ventricular BNP mRNA level under baseline conditions (without an elevated load).

### 3.3. *ET-1 and Ang II are not involved in the load-induced activation of c-fos gene expression*

Using the flow rate of 5 ml/min, the left ventricular *c-fos* mRNA levels were increased 1.5±0.2-fold compared to the baseline after two hours loading (Fig. 2,  $P < 0.05$ ). In contrast to the BNP response, the load-induced changes in *c-fos* gene expression were not affected by the drug treatments (Fig. 2). There were no significant treatment effects on *c-fos* mRNA levels in either loaded or unloaded mice hearts.

### *3.4. The effect of elevated coronary flow rate on left ventricular ET-1 and ANP gene expression.*

Since ET-1 appeared to be a major contributor to acute responses to elevated load in isolated perfused mice hearts, we next analyzed the changes in left ventricular preproET-1 mRNA levels after 2- hours loading. Left ventricular preproET-1 mRNA levels increased  $1.6 \pm 0.3$ -fold in response to mechanical loading (Fig. 3,  $P < 0.05$ ). This increase in ET-1 mRNA levels was unaffected by bosentan and CV-11974 treatments ( $1.8 \pm 0.2$  and  $2.4 \pm 0.4$ -fold increases compared to control, respectively,  $P = \text{NS}$  vs vehicle infused loaded hearts,  $P < 0.01$  vs vehicle infused control). Left ventricular ANP mRNA levels remained unchanged after acute, 2-hours loading (Fig. 3), in agreement with previous studies showing different regulation of ANP gene expression compared to BNP and *c-fos* [7, 15, 25, 26].

### *3.5. Effects of the drug treatments on perfusion pressure*

The perfusion pressure was continuously monitored to measure vasoactive effects of the drugs (Table 1). Since no differences were noted, we conclude that the changes in the gene expression by drug treatments did not arise from altered level of mechanical stimulation of the hearts. Moreover, at the end of the 2-hour experimental period, no differences were observed in the contractile performance of the hearts, as measured by developed tension (Table 1).

## **4. Discussion**

Our results show that the elevated coronary perfusion (Gregg phenomenon) is coupled with acute changes in gene expression in mice hearts. Furthermore, we show that the paracrine

regulators ET-1 and Ang II are the key mediators of the BNP gene expression response to load in mice hearts. The ET<sub>A</sub> receptor subtype is mainly responsible for mediating the effects of ET-1, since ET<sub>A</sub> antagonist BQ-123 produced similar attenuation of the load induced BNP gene expression as the mixed ET<sub>A/B</sub> antagonist bosentan. Under these experimental conditions, the regulation of *c-fos* gene expression in response to hemodynamic stress differed from that of BNP, being independent of both ET-1 and Ang II.

The activation of the early response genes in response to hemodynamic load is an early event in the series of changes leading to left ventricular hypertrophy and heart failure. Thus, understanding the molecular regulation of the early load-induced responses may potentially help to develop novel treatments for heart failure. *c-fos* and BNP are upregulated very early, while the induction of ANP gene expression in the overloaded left ventricle occurs later [25 – 28]. Mechanical stretch is coupled with the cellular release of Ang II and ET-1, which have been reported to act as chemical mediators of stretch-induced myocyte hypertrophy and BNP gene expression [8, 29]. Recently, a number of well-designed studies have provided further evidence supporting a role for cardiac ET-1 in LVH and heart failure in mice. Conditional cardiac overexpression of ET-1 was shown to lead to dilated cardiomyopathy and increased mortality in mice [30]. In agreement with this, cardiomyocyte-specific inactivation of ET-1 gene was able to prevent LVH after stimulation with tri-iodothyronine [31]. However, mice with cardiomyocyte-specific knockout of ET<sub>A</sub> receptor were not resistant to cardiac hypertrophy induced by Ang II or isoproterenol [32]. Thus, it appears that locally produced ET-1 plays a key role, but is not obligatory in the process of LVH and heart failure in mice.

Several experimental studies have also suggested a role for ET-1 and Ang II in the responses to acute cardiac overload [8, 29, 33 - 36]. Yet, other studies have shown that

they are not obligatory for acute induction of left ventricular gene expression or even for the development of LVH [9, 10, 32]. ET-1 and Ang II have been reported to potentiate each other's effects, and they may act in chain, Ang II inducing the ET-1 release [8, 37, 38]. Moreover, exogenously administered ET-1 and Ang II are potent stimuli of BNP gene expression and LVH [6, 39]. Although our present observation of ET-1 and Ang II dependence of left ventricular BNP gene expression response to load supports their role in the hypertrophic process in mice hearts, our data also shows that there is additional, bosentan- and CV-11974-insensitive mechanisms, involved in transducing mechanical load to responses in the gene expression; even combining the antagonists of both ET and Ang II systems did not block the BNP gene expression response completely, and they had alone or in combination no effect on *c-fos* gene expression. In the experimental model employed in the current study, ET-1 and Ang II also did not significantly potentiate each other's effects, as combined antagonism of both ET<sub>A/B</sub> and AT<sub>1</sub> receptors did not provide significant additional effect on the BNP gene expression responses compared to treatment with either antagonist alone.

Analogous to the gene expression responses, the paracrine mediators ET-1 and Ang II are important mediators of the contractile responses to load. In the rat, the slowly developing part of contractile response to stretch is mediated by ET-1 and Ang II, leading to activation of Na<sup>+</sup>/Ca<sup>2+</sup>- exchanger in reverse mode, increasing intracellular Ca<sup>2+</sup> as a final mechanism resulting in positive inotropism [40]. Similar mechanism suggesting a role for ET-1 and Ang II in the slow force response has been described in feline cardiac tissue [37]. On the other hand, in the ferret heart, the slow force response was mediated by ET-1 but not Ang II [41]. A recent study with strips from failing human myocardium showed that although a slow force response similar to the rat heart exists, Ang II and ET-1 are not obligatory mediators [42]. Earlier, the load induced slow force response in rabbit hearts has

also been reported to be independent of ET-1 and Ang II [43]. We have reported a significant contribution of ET-1 but not Ang II to contractile response associated with elevated coronary perfusion in mice hearts [18]. Thus, there appears to be potentially significant interspecies differences in the role of ET-1 and Ang II in acute cardiac responses to elevated load.

The present experimental model of cardiac overload utilizes the elevation of coronary flow rate as a loading stimulus. The elevation of coronary perfusion pressure stretches the left ventricular wall [11 - 14]. Since the wall stretch is a predominant determinant of BNP gene expression [6], the wall tension may contribute directly to the effect of elevated aortic perfusion pressure on cardiac gene expression. The primary event in the mechanosensing process in response to elevated rate of perfusion has been suggested to be the activation of the stretch activated ion channels, yet this is not thoroughly understood at present [44]. Our results support the model that the mechanical stimulus is able to activate ET-1 and Ang II signaling, which then are responsible in part, but not solely, for the load-responses at the gene expression level.

The elevation of coronary flow rate is likely to produce effects also on the function of endothelium, in addition to direct stretch on the ventricular wall. This hypothesis is supported by the ability of shear stress to activate multiple signaling cascades in endothelial cells [45, 46]. Furthermore, endothelial cells are able to respond to altered levels of stress by changing the secretion of vasoactive factors which are potent regulators of cardiac function including ET-1 [17, 47]. Capillary perfusion and ET-1 regulate contractile response to elevated coronary perfusion in different models [16, 18]. Underscoring the significance of ET-1 in cardiac responses to mechanical load, the ET-1 mRNA levels were increased in response to elevated coronary perfusion pressure. The present experimental model does not allow the analysis of the cell types responsible for the

expression of ET-1 (and Ang II). In myocardium, endothelial cells are the primary site of ET-1 production, but also fibroblasts, cardiomyocytes and even smooth muscle cells possess the ability to synthesize ET-1 [39]. While both ET-1 and Ang II are able to stimulate ET-1 gene expression [39], they did not account for the elevated level of preproET-1 mRNA based on the inability of bosentan and CV-11974 to attenuate the response. Thus, direct stretch of ventricular wall or shear stress induced by elevated coronary flow may explain elevated ET-1 observed in the present study. Previously, a transient increase in ET-1 mRNA levels in response to elevated shear stress in cultured endothelial cells has been reported [46, 47]. Furthermore, the long-term cardiac overload imposing stretch on the ventricular wall is a known stimulus for ET-1 synthesis and secretion [39]. The gene expression of the renin-angiotensin system components is regulated more slowly in response to load and thus it was out of the scope of the present study [48].

The regulation of *c-fos* gene expression appears to differ from that of BNP, since ET<sub>A/B</sub> or AT<sub>1</sub> receptor blockade had no effect on load induced increase of *c-fos* mRNA levels. This supports the concept that multiple pathways are involved in mechanotransduction process induced by cardiac overload. The differential regulation of BNP and *c-fos* may be related to the transcription factors responsible for activating the genes. An analysis of the promoter of the rat BNP gene has indicated the presence of a proximal activator element that is composed of an activator protein-1 (AP-1) like element, an M-CAT (transcription enhancer factor-1) and two GATA elements [28]. Recently, we have found in stretched culture of cardiomyocytes that mutation of GATA binding sequence of BNP promoter inhibited stretch-response by 40 %, and that it was almost completely abolished when GATA mutation was combined with Nkx-2,5 binding element mutation [49]. In contrast to BNP, the promoter region of the *c-fos* gene does not contain

any GATA binding sites [50], and the main transcription factors responsible for *c-fos* gene regulation are thought to be serum response factors and ternary complex factors including Elk-1, SAP-1 and cAMP response element binding protein [50].

Based on our results elevated mechanical load through increased coronary flow increases expression of BNP, *c-fos* and preproET-1 in mouse left ventricular myocardium. The increase of BNP mRNA was inhibited by ET-1 and Ang II receptor antagonists. Moreover, our study revealed an ET-1- and Ang II- independent component of the BNP gene expression in response to elevated coronary flow rate. In conclusion, these results provide evidence of the pivotal role of paracrine/autocrine mediators in gene expression response to elevated load in mice hearts.

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Table 1. Hemodynamic parameters in isolated mice hearts at the end of the experiments

	n	Heart rate, beats per minute	Perfusion pressure, mmHg	Developed tension, g
<b>Control</b>				
Vehicle	10	399±1	44±5	0.50±0.08
BQ-123	6	399±1	37±2	0.49±0.08
Bosentan	10	398±1	52±5	0.44±0.11
CV-11974	8	400±1	40±5	0.41±0.06
Bosentan + CV-11974	8	400±2	51±7	0.40±0.06
<b>Load</b>				
Vehicle	8	400±1	167±12	0.35±0.03
BQ-123	6	399±1	136±18	0.48±0.12
Bosentan	12	401±1	160±11	0.43±0.12
CV-11974	8	401±1	143±17	0.30±0.06
Bosentan + CV-11974	9	400±1	149±19	0.49±0.17

Values are mean ± SE. The parameters did not differ significantly from the respective vehicle treated group (Oneway ANOVA followed by a post hoc test for least significant differences).

