Effects of chronic cold exposure on the endothelin system


Cold temperatures have adverse effects on the human cardiovascular system. Endothelin (ET)-1 is a potent vasoconstrictor. We hypothesized that cold exposure increases ET-1 production and upregulates ET type A (ET$_A$) receptors. The aim of this study was to determine the effect of cold exposure on regulation of the ET system. Four groups of rats (6–7 rats/group) were used: three groups were exposed to moderate cold (6.7 ± 2°C) for 1, 3, and 5 wk, respectively, and the remaining group was maintained at room temperature (25°C) and served as control. Cold exposure significantly increased ET-1 levels in the heart, mesenteric arteries, renal cortex, and renal medulla. Cold exposure increased ET$_A$ receptor protein expression in the heart and renal cortex. ET type B (ET$_B$) receptor expression, however, was decreased significantly in the heart and renal medulla of cold-exposed rats. Cold exposure significantly increased the ratio of ET$_A$ to ET$_B$ receptors in the heart. An additional four groups of rats (3 rats/group) were used to localize changes in ET$_A$ and ET$_B$ receptors at 1, 3, and 5 wk of cold exposure. Immunohistochemical analysis showed an increase in ET$_A$, but a decrease in ET$_B$ receptor immunoreactivity in cardiomyocytes of cold-exposed rats. Increased ET$_A$ receptor immunoreactivity was also found in vascular smooth muscle cells of cold-exposed rats. Cold exposure increased ET$_A$ receptor immunoreactivity in tubule epithelial cells in the renal cortex but decreased ET$_B$ receptor immunoreactivity in tubule epithelial cells in the renal medulla. Therefore, cold exposure increased ET-1 production, upregulated ET$_A$ receptors, and downregulated ET$_B$ receptors.

CIH and other cold-related cardiovascular complications are not fully understood, although the involvement of the renin-angiotensin system (RAS) has been studied (43, 44, 46). The endothelin (ET) system plays an important role in regulation of blood pressure (BP), vascular tone, myocardial contractility, fluid balance, and hemodynamics (1, 15). The ETs are a group of vasoconstrictor peptides derived from vascular endothelial cells (9, 25, 51). Three ETs, ET-1, -2, and -3, all consisting of 21 amino acids, have been identified. ET-1, the predominant representative of the ET family, is the most potent natural mammalian vasoconstrictor agent yet discovered (31–41 times stronger than ANG II) (25) and is essential for cardiovascular regulation (1, 15, 35). The vasoconstrictive effect of ET-1 is mainly mediated by the endothelin type A (ET$_A$) receptor, which increases intracellular Ca$^{2+}$ concentration (28). ET-1 also has a vasodilatory effect (8), an action mediated by an ET type B (ET$_B$) receptor in endothelial cells (ET$_B1$). ET$_B2$ receptors have been found in vascular smooth muscle cells (VSMC) and can mediate vasoconstriction in multiple blood vessels, primarily veins. Although the discrete function and location of ET$_B$ receptors were proposed in humans, these two subtypes of ET$_B$ receptors have not been cloned in rats. Thus ET$_B$ receptors are quantified as a whole in rats.

An increase in the production of ET-1, but not ET-2 or ET-3, has been reported to contribute to the development of hypertension (1, 3, 15, 31, 49, 52). The ET system may be involved in human hypertension (35, 49). For example, ET-1 plays a role in salt-sensitive hypertension (1, 27, 29, 31). Mice with cardiomyocyte-specific ET-1 disruption did not develop hyperthyroid cardiac hypertrophy (38). Blockade of ET receptors attenuated cardiac hypertrophy and end-organ damage in hypertensive rats (11). It seems that ET$_A$ receptors contribute to the pathogenesis of hypertension, whereas ET$_B$ receptors protect against vascular and renal injury (29). Therefore, ET-1 knockout in the renal collecting duct, a region of high ET$_B$ receptor expression, results in hypertension (3).

Although ET-1 is produced predominantly by endothelial cells, it also is generated in cardiomyocytes, VSMC, and renal tubule epithelial cells (15, 23, 25). The ET system exerts a broad range of actions on these tissues that modulate BP and control extracellular fluid volume. ET-1 is a local hormone, and its distribution and function may vary with tissues in response to environmental stimuli. It is not known, however,
whether changes in the activity of the ET system and in the regulation of ET receptors occur during chronic cold exposure. This information is critical for understanding the contribution of the ET system to cold-induced cardiovascular complications. We hypothesized that cold exposure increases ET-1 production and upregulates ETA receptors in cardiovascular and renal tissues. Thus the purpose of this experiment was to determine whether and to what extent cold exposure alters the ET system by assessing the production of ET-1 and the regulation of ET receptors at 1, 3, and 5 wk of cold exposure.

METHODS

Animals and experimental protocols. The study was carried out according to the guidelines of the National Institutes of Health on the care and use of laboratory animals. The project, of which this study was a part, was approved by the Institutional Animal Care and Use Committee.

Twenty-five male Harlan Sprague Dawley rats [180 – 200 g body wt (BW)] were divided into four groups (6 or 7 rats/group). All animals were housed individually, and standard rat Chow and tap water were provided. BP and BW were measured twice during a 1-wk control period at room temperature (RT, 25°C, warm control). Systolic BP was measured from the tail of each unanesthetized rat by the tail-cuff method, with slight warming (28°C), but not heating, of the tail. All rats were handled frequently (2 twice per day) to minimize handling stress. Animals did not appear stressed during BP measurement. The tail-cuff method has been commonly used by us (43–45) and others (33, 55) to delineate cold-induced elevation of BP. Intra-arterial cannulation has confirmed that the noninvasive tail-cuff method is effective and reliable in monitoring syctic BP in rats exposed to cold (11, 40).

After the control period, three groups (6 rats/group) were moved into a cold climate-controlled walk-in chamber (6.7°C) and served as a control. Relative humidity was controlled automatically and maintained at 45 ± 5% in both thermal environments. Our previous studies showed that rats are able to maintain their core temperatures during exposure to moderate cold (48). BP and BW were measured weekly during exposure to cold. At 1 and 3 wk of exposure to cold, one cold-exposed group and two rats from the warm control (RT, 25°C) group were killed by decapitation. At 5 wk of exposure to cold, the last group from the cold environment and three rats from the warm control (RT, 25°C) group were killed. Although the control rats were killed at different times, they were considered one control (warm control) group (7 rats/group) for the purpose of statistical analysis, because there were no significant changes in biochemical measurements (e.g., ET-1, prepro-ET-1 mRNA, and ET receptor expression) among the three time points. Blood was collected in vials containing 200 μl of 0.1 M EDTA and centrifuged at 4°C (1,000 g) for 15 min. Plasma was collected for measurement of plasma ET-1. The heart and kidneys were removed, cleaned, and weighed. The heart, kidneys, and third-order and smaller branches of superior and inferior mesenteric arteries were removed for measurement of tissue ET-1 and ET receptor protein expression. Third-order and smaller branches of mesenteric arteries are regarded as resistance vessels (18, 50).

ET-1 measurements. Tissue samples were weighed and homogenized in 1.0 M acetic acid with 0.000015 M pepstatin for 1 min. The homogenate was centrifuged at 4°C (20,000 g) for 30 min. The supernatant was stored at −80°C. ET-1 levels in the heart, kidneys, mesenteric arteries, and plasma were measured using the ET-1 (human) TiterZyme immunoassay kit (Assay Design) according to the manufacturer’s instruction.

Western blot. Western blot analysis was performed as described previously (46, 52). Briefly, the membrane was blocked with 5% fat-free milk for 30 min and then incubated in rabbit anti-ETA antibody (1:200 dilution) or rabbit anti-ETB antibody (1:200 dilution; Alomone Labs, Jerusalem, Israel) for 12–18 h. These antibodies have been confirmed, by the manufacturer and other investigators, to be specific for rat ETA and ETB receptors, respectively (19). The membrane was then incubated for 1 h (ETA) or 2 h (ETB) with a secondary antibody, goat anti-rabbit antibody labeled with horseradish peroxidase (1:1,000 dilution). The slices were blocked with 10% goat serum albumin for 1 h and incubated with rabbit anti-ET_A or rabbit anti-ET_B (1:200 dilution) antibody for 1 h. Staining was visualized with a horseradish peroxidase system (DakoCytomation). Digital photographs were taken using a Zeiss microscope and imaging software (SPOT).

Statistical Analysis

BP and BW data were analyzed by one-way ANOVA for repeated measurements (in time). The data for ET-1 concentration, organ weights, and ratio of ETA to ETB receptor protein expression were analyzed by one-way ANOVA. Newman-Keuls procedure was used to assess the significance of differences between means. Significance was set at the 95% confidence limit.

RESULTS

Effects of Cold Exposure on BP and Heart Weight

BP did not differ significantly among the four groups during the control period at RT (Fig. 1). BP of rats exposed to 6.7°C increased significantly as early as 1 wk of exposure to cold. BP reached 162.89 ± 3.6 mmHg at 5 wk of exposure to cold. The warm control group maintained a steady BP, averaging 112.3 ± 4.2 mmHg, over the 5-wk period at RT (Fig. 1).

All cold-exposed rats appeared healthy, with a normal rate of weight gain (data not shown). Chronic cold exposure significantly increased heart weight (Fig. 2A) and kidney weight (Fig. 2B). Heart and kidney weights did not differ significantly between 1 and 5 wk of exposure to cold, indicating that cardiac and renal hypertrophy were not further exacerbated with increased duration of cold exposure.

Effects of Cold Exposure on ET-1 Production

The immunoreactive ET-1 level in mesenteric resistance arteries increased significantly at 1 wk of exposure to cold (Fig. 3B). ET-1 in the cold-exposed rats increased approximately fourfold compared with the rats maintained at RT (warm control). However, the ET-1 level in mesenteric arteries began to decrease at 3 wk of exposure to cold and returned to the
control level by 5 wk of cold exposure. The ET-1 level in mesenteric arteries remained elevated at 3 wk of cold exposure ($P < 0.001$ vs. warm control), although it was decreased significantly compared with 1 wk of cold exposure (Fig. 3B). In contrast, ET-1 levels in the heart did not increase significantly until 5 wk of exposure to cold (Fig. 3A). Total kidney ET-1 content increased significantly as early as 1 wk of exposure to cold and continued to increase throughout cold exposure (Fig. 3C). The ET-1 level in the renal cortex increased significantly during the first 3 wk and reached to the highest level by 5 wk of cold exposure (Fig. 3D). However, the ET-1 level in the renal medulla did not change significantly during the first 3 wk of exposure to cold but increased significantly by 5 wk of cold exposure (Fig. 3E). Plasma ET-1 did not change significantly throughout cold exposure (Fig. 3F).

**Effects of Cold Exposure on ET Receptor Protein Expression**

Western blot analysis indicated that ETA receptor protein expression in the heart was increased significantly at 3 wk of exposure to cold ($P < 0.01$) and was further increased ($P < 0.001$) by 5 wk of cold exposure (Fig. 4, A and B). In contrast, ETB receptor protein expression in the heart was decreased significantly ($P < 0.001$) as early as 1 wk of exposure to cold and remained at a low level throughout cold exposure (Fig. 4, A and C). Cold exposure decreased ETB receptor expression $\sim$10-fold. The ratio of ETA to ETB receptor expression was $<1$ at RT (warm control; Fig. 4D). Chronic cold exposure significantly increased the ratio of ETA to ETB receptor expression. The ratio of ETA to ETB receptor expression was about 4:1 at 1 wk of exposure to cold and increased to 6:1 by 5 wk of cold exposure (Fig. 4D).

ETA receptor expression in the renal cortex increased significantly at 3 wk of exposure to cold and further increased at 5 wk of cold exposure ($P < 0.001$) compared with control rats maintained at RT (warm control; Fig. 5A). ETB receptor protein expression was not detectable in the renal cortex. However, ETB receptor expression in the renal medulla was abundant in rats maintained at RT (warm control). ETB receptor expression was decreased significantly by 1–5 wk of cold exposure (Fig. 5B). ETA receptor expression was not detectable in the renal medulla.
Immunohistochemical Analysis of ET Receptor Expression

ET_A receptor immunostaining was barely detectable in the left ventricle of rats maintained at RT (warm control; Fig. 6A). ET_A receptor immunoreactivity was increased by 1 wk of exposure to cold and further increased with the duration of cold exposure. The strongest ET_A receptor immunoreactivity in the left ventricle was found at 5 wk of cold exposure (Fig. 6A). The increased ET_A receptor immunoreactivity was localized in cardiomyocytes and VSMC. In contrast, strong ET_B receptor immunostaining was found in cardiomyocytes of rats maintained at RT (warm control; Fig. 6A). The increased ET_A receptor immunoreactivity was localized in cardiomyocytes and VSMC. In contrast, strong ET_B receptor immunoreactivity was decreased by 1 wk of exposure to cold and remained at a low expression level throughout cold exposure (Fig. 6B). ET_B receptor immunoreactivity was weaker in VSMC and endothelial cells than in myocytes.

ET_A receptor immunostaining was barely detectable in the renal cortex of rats maintained at RT (warm control; Fig. 7A). ET_A receptor immunoreactivity was increased in the renal cortex at 3–5 wk of cold exposure (Fig. 7A). The increased ET_A receptor expression was found in glomerular VSMC and tubule epithelial cells of cold-exposed rats. In contrast, ET_B receptors were mainly localized in renal tubule epithelial cells, with strong expression in rats maintained at RT (warm control; Fig. 7B). ET_B receptor immunoreactivity was decreased greatly by 1–5 wk of cold exposure.

DISCUSSION

The present data clearly showed that chronic cold exposure increased ET-1 levels in cardiovascular tissues. The most dramatic increase in ET-1 occurred in mesenteric resistance arteries as early as 1 wk of exposure to cold, when BP began to rise. These results suggest, but do not prove, that ET-1 contributes to the initiation of CIH. However, chronic cold exposure did not change plasma levels of ET-1. On the other hand, acute (minutes or hours) cold exposure increases plasma ET-1 levels (24, 26). Thus plasma ET-1 responds differently to...
Fig. 4. A: Western blot analysis of ET type A and B (ET<sub>A</sub> and ET<sub>B</sub>) receptor expression in left ventricle (50 μg/lane of total protein) of rats maintained at room temperature (warm control) and rats exposed to cold for 1, 3, and 5 wk. B and C: optical density of ET<sub>A</sub> and ET<sub>B</sub> receptor expression in heart. D: ratio of ET<sub>A</sub> to ET<sub>B</sub> receptor density in heart. ODu, optical density unit. Values are means ± SE (n = 6–7). **P < 0.01; ***P < 0.001 vs. warm control. +++P < 0.01; ++++P < 0.001 vs. cold 1 week.

Fig. 5. Western blot analysis of ET<sub>A</sub> receptor expression in renal cortex (50 μg/lane of total protein; A) and ET<sub>B</sub> receptor expression in renal medulla (50 μg/lane of total protein; B) in rats maintained at room temperature (warm control) and rats exposed to cold for 1, 3, and 5 wk. Values are means ± SE (n = 6–7). **P < 0.01; ***P < 0.001 vs. warm control. +++P < 0.01; ++++P < 0.001 vs. cold 1 week.
acute and chronic cold exposure. It should be emphasized that ET-1 is produced in endothelial cells and is predominantly secreted toward the adjacent VSMC, supporting the notion that ET-1 is an autocrine/paracrine agent, rather than a circulating hormone. Thus tissue levels of ET-1 are more important than plasma levels of ET-1 in assessing the contribution of the ET system to CIH. Physiologically, an increase in BP inhibits vascular ET formation. Therefore, the cold-induced increase in ET-1 production, at least during the early stage of cold exposure, is not due to hypertension-associated endothelial damage, because CIH is not fully established until 5 wk after exposure to cold (39). Thus the cold-induced increase in ET-1 production may be due to endocrine changes associated with cold exposure. It has been reported that ANG II is an important stimulus for the production of ET (4, 6, 13, 49). Our previous studies indicated that cold exposure activates the RAS (42, 45, 46). Therefore, the hypothesis that the cold-induced activation of the ET system is mediated by the RAS warrants further investigation.

Our previous studies indicated that cold-induced cardiac hypertrophy was independent of high BP, because prevention or reduction of CIH failed to attenuate the development of...
cardiac hypertrophy (41, 44, 45). It has been reported that the ET system can contribute to cardiac hypertrophy (7, 16, 20, 29, 34). ET-1 added directly to cardiomyocytes in culture increases the size of the cells and increases actin production (17). The most recent report indicates that ET-1 produced locally by cardiomyocytes is an important mediator for myocardial hypertrophy induced by thyroid hormone (38). Although ET-1 levels in the heart were not elevated until 5 wk of exposure to cold, the ratio of cardiac ET<sub>A</sub> to ET<sub>B</sub> receptors was markedly increased as early as 1 wk of exposure to cold. It was reported that ET<sub>A</sub> receptor-mediated action plays an important role in the pathogenesis of deoxycorticosterone acetate-salt-induced hypertension and cardiac hypertrophy (29). However, the ET<sub>B</sub> receptor-mediated action protects against vascular and end-organ damage in this model of hypertension. An increase in the ET<sub>A</sub>-to-ET<sub>B</sub> receptor ratio points out the necessity to determine whether the alteration in the ET system in the heart is involved in cold-induced cardiac hypertrophy.

The kidney is one of the most important organs in the regulation of systemic hemodynamics and is a key target organ for ET-1 as well as a major site of ET-1 production. In addition to its effect on renal hemodynamics, ET-1 directly regulates tubular handling of electrolytes and water (1). The cold-induced increase in renal ET-1 occurred predominantly in the renal cortex. An increase in ET-1 levels in the renal cortex decreases renal blood flow and glomerular filtration rate (1, 10), resulting in antidiuresis and antinatriuresis. This effect is mediated by cortical vasoconstrictor effects of ETA receptors, which were also increased by cold exposure. In the renal medulla, however, ET-1 inhibits Na<sup>+</sup>-K<sup>+</sup>-ATPase activity and blocks the stimulatory effects of vasopressin on water reabsorption and, thereby, induces natriuresis and diuresis (1, 22, 23). This effect is mediated by ET<sub>B</sub> receptors through increases in nitric oxide release and cellular cGMP levels (2). Thus the effects of ET-1 on Na<sup>+</sup> and water reabsorption in the medulla are different from those in the cortex. ET-1 levels in the medulla did not increase until 5 wk, but medullary ET<sub>B</sub> receptor protein expression was decreased throughout exposure to cold. Thus the differential regulation of the ET system in the cortex and medulla tends to increase reabsorption of Na<sup>+</sup> and water, thereby causing the fluid retention that is seen in cold-exposed rats (40). In addition, ET-1 has proliferative effects that may contribute to cold-induced renal hypertrophy. The present data also demonstrated that the renal cortex was predominantly occupied by ET<sub>A</sub> receptors, whereas in the renal medulla ET<sub>B</sub> receptors were heavily distributed throughout the tubules.

The cold-induced downregulation of ET<sub>B</sub> receptors in the heart and renal medulla was probably due to increased ET-1 levels in these tissues. However, cold exposure upregulated ET<sub>A</sub> receptors in the heart and renal cortex against the background of unchanged or increased ET-1. The mechanism responsible for this unique regulation of ET<sub>A</sub> receptors during cold exposure is still under investigation. Recent advances in receptor physiology indicate that a receptor can be regulated by many factors in addition to its ligand (32, 42, 43, 46, 48, 54). It is known that cold exposure increases the secretion of thyroid hormones, which may activate the cardiac ET system (38). Therefore, it is worthwhile to test whether thyroid hormones mediate the cold-induced upregulation of ET<sub>A</sub> receptors.

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REFERENCES

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