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Possible involvement of oxidative stress in hypoxia-induced adrenomedullin secretion in cultured rat cardiomyocytes

Fumiki Yoshihara [a,*], Takeshi Horio [b], Toshio Nishikimi [c], Hisayuki Matsuo [a], Kenji Kangawa [a]

[a] *Research Institute, National Cardiovascular Center, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan*
[b] *Division of Hypertension and Nephrology, National Cardiovascular Center, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan*
[c] *Department of Cardiovascular Medicine, Dokkyo Medical School, Tochigi 321-0293, Japan*

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Abstract

Although hypoxia induces adrenomedullin gene expression in cultured rat cardiac myocytes, it is still unknown whether oxidative stress is involved in the hypoxia-induced adrenomedullin production. We investigated whether oxidative stress might participate in hypoxia-induced adrenomedullin secretion and whether adrenomedullin might have a protective effect on damaged myocytes. Hypoxia increased adrenomedullin secretion and its gene expression in cardiac myocytes, but not in nonmyocytes. Furthermore, oxidative stress (hydrogen peroxide) also increased adrenomedullin secretion from myocytes. *N*-acetyl-L-cysteine, a free radical scavenger, completely inhibited the stimulation of adrenomedullin secretion by hydrogen peroxide, and this agent reduced the stimulation of adrenomedullin secretion by hypoxia. Lactate dehydrogenase leakage, a marker of cell injury, was significantly increased with the exposure to hydrogen peroxide and adrenomedullin significantly reduced this leakage. These findings suggest that an oxidative stress may be involved, in part, in the increased adrenomedullin secretion from cardiac myocytes under hypoxic condition. Adrenomedullin secreted from myocytes may play a cell protective role in an autocrine manner.

Keywords: Hypoxia; Oxidative stress; Adrenomedullin; Cardiomyocyte

1. Introduction

We previously reported that adrenomedullin was synthesized and secreted from cultured rat cardiac myocytes and fibroblasts (Horio et al., 1998) and that adrenomedullin increased cAMP in both two types of cardiac cells (Nishikimi et al., 1998). Furthermore, the synthesis of adrenomedullin in myocytes and fibroblasts was increased after stimulation with interleukin-1β (Horio et al., 1998), and the secreted adrenomedullin might play a role in the process of cardiac remodeling through its suppressive effect of collagen synthesis (Horio et al., 1999). Recent studies demonstrated that adrenomedullin transcription could be induced by hypoxia and that this response was mediated by hypoxia-inducible factor-1 (HIF-1) consensus sites in adrenomedullin promoter (Cormier-Regard et al., 1998; Nguyen and Claycomb, 1999). In addition, not only hypoxia but also oxidative stress augments the release of adrenomedullin into the medium and the adrenomedullin gene expression in cultured vascular smooth muscle cells (Ando et al., 1998; Nagata et al., 1999) and endothelial cells (Nakayama et al., 1999; Chun et al., 2000). Furthermore, previous reports demonstrated that oxidative stress participated in the pathophysiology of myocardial infarction (Theres et al., 2000; Mansuy et al., 2000), and that the adrenomedullin concentration and its gene expression in the infarcted myocardium were increased (Nagaya et al., 2000), suggesting that oxidative stress might be one of the stimulators of adrenomedullin production in the infarcted myocardium. In addition, hypoxia increased the mitochondrial reactive oxygen species generation in cardiomyocytes and this response might be mediated by hypoxia-induced suppression of cytochrome oxidase (Duranteau et al., 1998). Thus, the present study sought to test the hypothesis that oxidative stress is involved in adrenomedullin release from cardiac myocytes under hypoxic condition, and that oxidative stress itself is a stimulator of adrenomedullin release from cardiomyocytes.

* Corresponding author. Tel.: +81-6-6833-5012; fax: +81-6-6872-8091.
E-mail address: fyoshi@ri.ncvc.go.jp (F. Yoshihara).

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2. Materials and methods

This study was performed in accordance with the guidelines of the Animal Care Committee of the National Cardiovascular Center Research Institute.

2.1. Cell culture

Primary cultures of neonatal ventricular myocytes and nonmyocytes were prepared as described previously (Horio et al., 1998; Nishikimi et al., 1998). Briefly, apical halves of cardiac ventricles from 1- to 2-day-old Wistar rats were separated, minced, and dispersed with 0.1% collagenase type II (Worthington Biochemical). To segregate myocytes from nonmyocytes, a discontinuous gradient of Percoll (Sigma) was prepared. After centrifugation, the upper layer consisted of a mixed population of nonmyocyte cell types, and the lower layer consisted almost exclusively of cardiac myocytes. Both myocytes and nonmyocytes were washed twice by centrifugation and resuspension to remove all traces of Percoll. After the myocytes were incubated twice on uncoated 10-cm culture dishes for 30 min to remove any remaining nonmyocytes, the nonattached viable cells were plated on gelatin-coated 6-well culture plates (4.0×10^5 cells/well) and then cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) supplemented with 10% fetal calf serum (Life Technologies). Nonmyocyte cells were resuspended in DMEM with 10% fetal calf serum and plated onto uncoated 10-cm culture dishes for 30 min. After the plating period, nonadherent cells and debris were washed away, and fresh medium was added. Cells were allowed to grow to confluence, trypsinized, and passaged 1:3. This procedure yielded cultures of cells that were almost exclusively fibroblasts by first passage as previously described (Villarreal et al., 1993). Nonmyocytes at the second or third passage were plated onto 6-well plates and allowed to grow to confluence (2.5×10^5 cells/well). After incubation in DMEM with fetal calf serum, the culture medium was changed to serum-free DMEM, and all experiments were performed 24 h later.

2.2. Hypoxia induction

To induce hypoxia, we used the NAPCO 7001 series water-jacketed, microprocessor-controlled CO_2 incubator according to the manufacturer's instructions. Cardiac myocytes and nonmyocytes were cultured in the incubator with 5% CO_2/94% N_2/1% O_2 as the hypoxic condition (1% O_2).

2.3. Oxidative stress and antioxidant agent

Cardiac myocytes were treated with 10^{-5}–10^{-4} M hydrogen peroxide (H_2O_2) for the indicated time periods. Effect of an antioxidant was examined by adding 10^{-4}–10^{-2} M *N*-acetyl-L-cysteine (Sigma) with hypoxia or H_2O_2.

2.4. Measurement of immunoreactive adrenomedullin and atrial natriuretic peptide (ANP)

The culture medium was acidified with acetic acid, boiled for 5 min to inactivate intrinsic proteases, and lyophilized. The radioimmunoassay for rat adrenomedullin or rat ANP was performed as previously reported (Nishikimi et al., 1997; Aburaya et al., 1989). The anti-adrenomedullin antibody recognized the C-terminal region of rat adrenomedullin and did not cross-react with rat ANP. The antibody against rat ANP also did not cross-react with rat adrenomedullin. These assays were performed in duplicate.

2.5. Northern blot analysis for rat adrenomedullin and ANP mRNA

Total RNA was extracted from cultured cells by the acid guanidinium thiocyanate–phenol–chloroform method, according to the method previously reported (Sugo et al., 1994). Total RNA was denatured with formaldehyde and formamide, and electrophoresed on a 1% agarose gel containing formaldehyde. RNA in the gel was then transferred to a nylon membrane (Zeta-Probe blotting membrane, Bio-Rad Laboratories) and fixed by ultraviolet irradiation. Hybridization and washing of the membrane were carried out with cDNA probes for rat adrenomedullin and rat ANP genes as described previously (Yoshihara et al., 2000). Band intensity was estimated using a radioimage analyzer (BAS-5000, Fuji Photo Film). For comparison of mRNA contents in each sample, the same membrane was rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe.

2.6. Measurement of lactate dehydrogenase (LDH)

Loss of cardiac myocyte integrity was evaluated spectrophotometrically by measurement of the LDH activity into the supernatant, using a standard kit (LDH-Cytotoxic Test, Wako).

2.7. Calculations and statistical analysis

All values are given as the mean ± S.D. ($n = 3$–6 in each group). The statistical significance of differences in the

Table 1
Basal secretion of adrenomedullin from cultured cardiac myocytes and nonmyocytes

	Adrenomedullin (fmol/10^5 cells)			
	12 h	24 h	48 h	72 h
Myocytes	8.8 ± 1.2	10.7 ± 1.8	15.7 ± 2.5	17.7 ± 2.9
Nonmyocytes	17.3 ± 2.5	18.7 ± 2.8	23.1 ± 2.8	27.5 ± 5.8

Values are given as the mean ± S.D. of six measurements ($n = 6$ in each group).

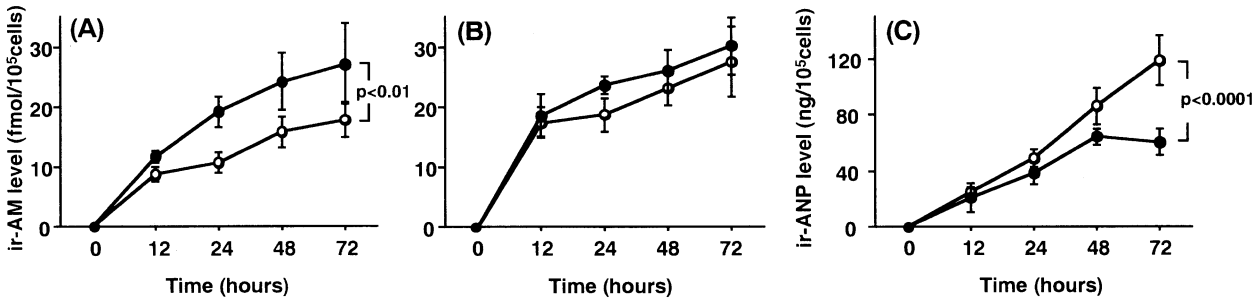

Fig. 1. Time course of the effects of hypoxia on the secretion of adrenomedullin in cultured cardiac myocytes (A) and nonmyocytes (B) and on the secretion of ANP in cardiac myocytes (C) under normoxic (open circle) and 1% O_2 hypoxic (closed circle) conditions. Values are mean ± S.D. ($n = 6$ in each group).

time course of adrenomedullin and ANP between the two groups was evaluated using a repeated measure-analysis of variance (ANOVA). The multiple comparison was performed with a one-way ANOVA followed by Scheffe's method. A value of $P < 0.05$ was accepted as statistically significant.

3. Results

3.1. Effects of hypoxia on the secretion of adrenomedullin and the expression of adrenomedullin mRNA in cardiac myocytes and nonmyocytes

Both myocytes and nonmyocytes secreted adrenomedullin into medium in a time-dependent manner (Table 1). In cardiac myocytes, the secretion of adrenomedullin was significantly induced, whereas the adrenomedullin secretion from nonmyocytes was not increased under the hypoxic condition (Fig. 1A,B). The expression of adrenomedullin mRNA was significantly increased at 24 and 48 h after the initiation of hypoxic condition in myocytes, but not in nonmyocytes (Fig. 2A,B).

3.2. Effects of hypoxia on the secretion of ANP and the expression of ANP mRNA in cardiac myocytes

As shown in Figs. 1C and 2C, cultured neonatal rat cardiac myocytes secreted a high amount of ANP in a time-dependent manner, and strong gene expression on ANP was observed in those cells. In contrast with the increase of adrenomedullin secretion and adrenomedullin gene expres-

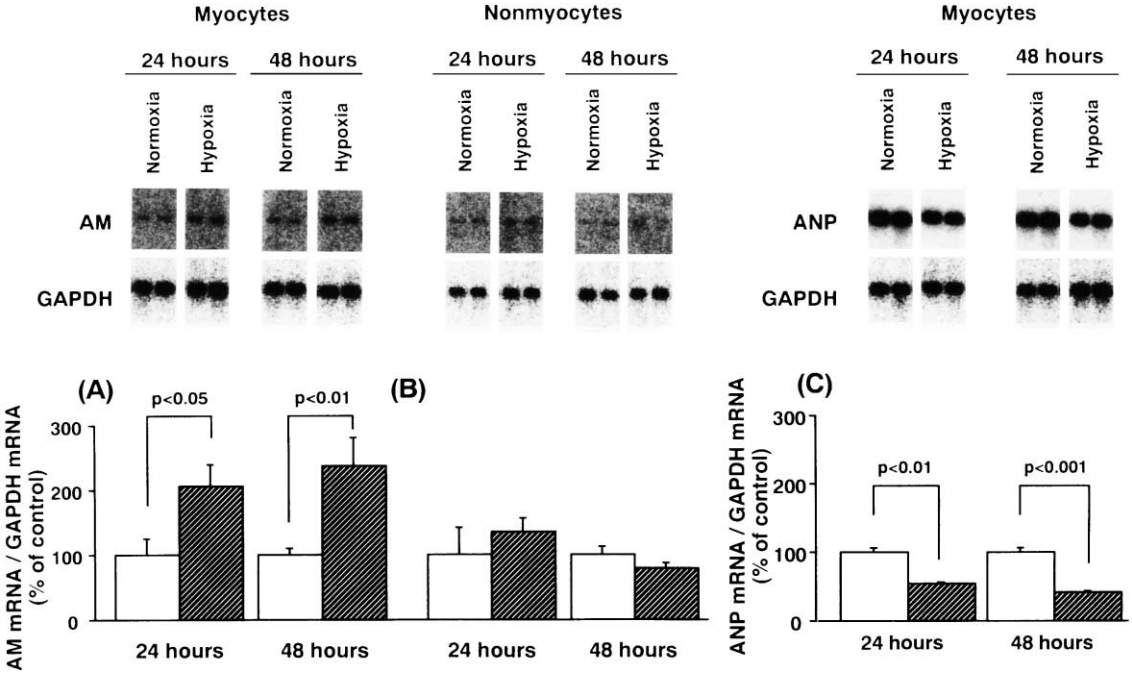

Fig. 2. Representative image and quantitative analysis of the expression of rat adrenomedullin and GAPDH mRNA in cultured cardiac myocytes (A) and nonmyocytes (B) and of the expression of rat ANP and GAPDH mRNA in cardiac myocytes (C) with (hatched bars) or without (open bars) hypoxia exposure. Values are mean ± S.D. ($n = 3$ in each group).

sion in cardiac myocytes, the secretion of ANP from cardiac myocytes was significantly reduced under the hypoxic condition (Fig. 1C), and the gene expression of ANP was also significantly decreased at 24 and 48 h after the initiation of the hypoxic condition in myocytes (Fig. 2C).

3.3. Effects of oxidative stress and antioxidant agent on adrenomedullin secretion in cardiac myocytes

The adrenomedullin secretion in myocytes was significantly increased by 10^{-4} M H_2O_2 (+70%), whereas its secretion showed no change under 10^{-5} M H_2O_2 (Fig. 3A). The adrenomedullin secretion induced by 10^{-4} M H_2O_2 in myocytes was almost completely inhibited by 10^{-3} and 10^{-2} M *N*-acetyl-L-cysteine (Fig. 3B). In addition, *N*-acetyl-L-cysteine significantly inhibited the adrenomedullin secretion induced by hypoxia (1% O_2) in myocytes (Fig. 3C). The inhibition by 10^{-3} and 10^{-2} M *N*-acetyl-L-cysteine of hypoxia-induced adrenomedullin secretion was −41% and −45%, respectively.

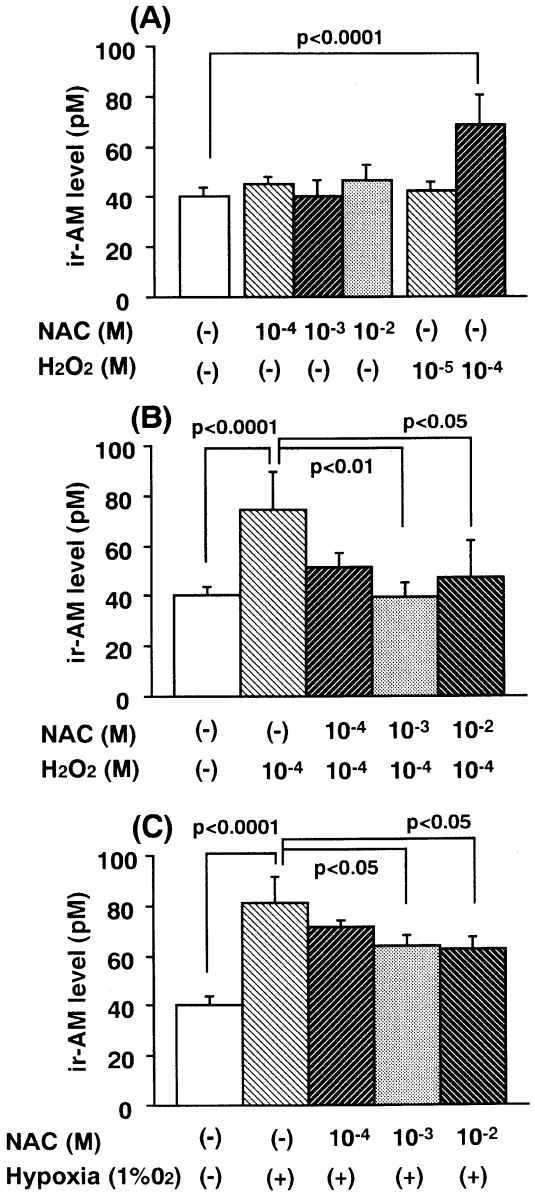

Fig. 3. (A) Effects of *N*-acetyl-L-cysteine (NAC) or H_2O_2 on adrenomedullin secretion in cultured cardiac myocytes. (B) Effects of NAC on the H_2O_2-induced increase in adrenomedullin secretion in cultured cardiac myocytes. (C) Effects of NAC on the hypoxia-induced increase in adrenomedullin secretion in cultured cardiac myocytes. Values are mean ± S.D. ($n=6$ in each group).

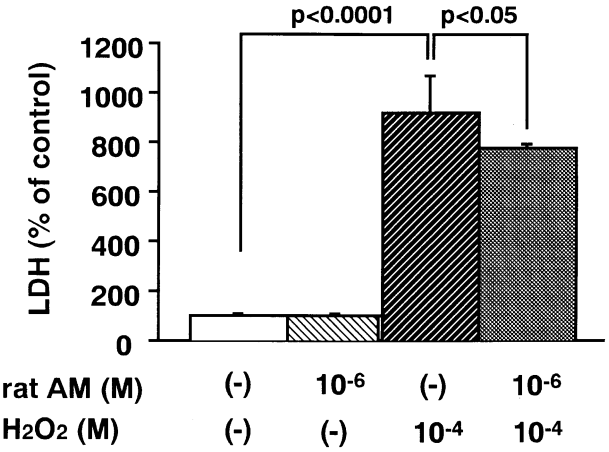

Fig. 4. Effects of rat adrenomedullin on the H_2O_2-induced LDH leakage into the medium from cultured cardiac myocytes. Values are mean ± S.D. ($n=6$ in each group).

3.4. Effect of adrenomedullin on LDH leakage from cardiac myocytes

As shown in Fig. 4, 10^{-4} M H_2O_2 markedly increased LDH leakage into the medium from cardiac myocytes, whereas 10^{-6} M rat adrenomedullin partially but significantly (−17%) suppressed the LDH leakage induced by 10^{-4} M H_2O_2.

4. Discussion

The present study was designed to examine whether hypoxia modulated adrenomedullin and ANP secretion from purified cardiac ventricular myocytes, and whether oxidative stress was involved in the hypoxia-induced modulation of adrenomedullin secretion from ventricular myocytes. We also evaluated the LDH leakage in the culture medium as an index of oxidative stress-induced cell injury. We demonstrated for the first time that hypoxia induced adrenomedullin secretion and suppressed ANP in a time-dependent manner and that the oxidative stress also induced adrenomedullin secretion from ventricular myocytes and *N*-acetyl-L-cysteine, a free radical scavenger partially but significantly inhibited the stimulation of adrenomedullin secretion by hypoxia from ventricular myocytes. The LDH measurements revealed that adrenomedullin signifi-

cantly reduced the oxidative stress-induced increase of LDH leakage in the culture medium, suggesting that adrenomedullin secreted from ventricular myocytes might have a protective effect on damaged myocytes due to oxidative stress.

Adrenomedullin gene expression and its secretion from purified cardiac ventricular myocytes were increased under hypoxic conditions, whereas ANP gene expression and its secretion were decreased in the present study. Previous studies also demonstrated that the exposure of cultured ventricular myocytes to hypoxia resulted in an increase of adrenomedullin mRNA level (Cormier-Regard et al., 1998); however, the influence of hypoxia on ANP gene expression and its secretion from cultured neonatal rat cardiac ventricular myocytes has never been elucidated. In cultured AT-1 cells, which were obtained from a transplantable mouse atrial cardiomyocyte tumor lineage, hypoxia augmented ANP gene expression and its secretion (Chen et al., 1997). These cells have been reported to retain a pattern of gene expression characteristic of normal adult mouse myocytes and to express genes coding for adult protein isoforms (Claycomb et al., 1998). These findings suggest that the difference of cell sorts and characteristics may be one of the causes of the different cell reaction, such as ANP gene expression and its secretion due to hypoxic stimulation between the primary cultured neonatal rat cardiac ventricular myocytes and the AT-1 cells.

Hypoxia is known to stimulate adrenomedullin gene expression in cardiac myocytes (Cormier-Regard et al., 1998). Furthermore, hypoxia generates intracellular reactive oxygen species (Duranteau et al., 1998). These findings suggest that the augmented adrenomedullin gene expression in cardiomyocytes under hypoxic condition may be the consequence of the stimulation of reactive oxygen species generation by hypoxia. However, the previous reports demonstrated that the adrenomedullin gene in the cardiac myocytes is up-regulated by hypoxia via the HIF-1 system (Cormier-Regard et al., 1998; Nguyen and Claycomb, 1999). The present study demonstrated that the oxidative stress was involved, in part, in the increased adrenomedullin secretion under hypoxic conditions, suggesting that the hypoxia-induced adrenomedullin secretion might be partially mediated by not only the HIF-1 system but also the stimulation of reactive oxygen species generation. Furthermore, H_2O_2 activates the nuclear factor-κB (NF-κB) pathway in cardiac myocytes (Norman et al., 1998). We previously reported that adrenomedullin secretion from cardiac myocytes was augmented by interleukin-1β (Horio et al., 1998). In addition, interleukin-1β generates intracellular reactive oxygen species and induces consequent activation of NF-κB. These findings suggest that the augmented adrenomedullin secretion by reactive oxygen species may be the consequence of the activation of the NF-κB pathway. The human adrenomedullin gene shows the presence of NF-κB binding motifs in its promoter region (Ishimitsu et al., 1994), supporting this hypothesis. The significance of the stimulation of reactive oxygen species generation in the inducible character of adrenomedullin observed in response to hypoxia should be examined in future studies.

In the present study, we reported that adrenomedullin significantly suppressed H_2O_2-induced LDH leakage in the culture medium, suggesting that adrenomedullin suppresses H_2O_2-induced myocyte injury. Previous studies demonstrated that adrenomedullin inhibited endothelial cell apoptosis (Kato et al., 1997) and its mechanism was reported to be mediated by the nitric oxide effect (Sata et al., 2000) and/or the upregulation of the max gene which is a heterodimeric partner of c-*myc* (Shichiri et al., 1999). In addition, H_2O_2 is an inducer of myocardial cell apoptosis (Cook et al., 1999). These findings suggest that the antiapoptotic effect of adrenomedullin may contribute to its suppression of H_2O_2-induced LDH leakage in the culture medium. Oxidative stress is an aggravating factor in the pathophysiology of acute myocardial infarction (Theres et al., 2000; Mansuy et al., 2000). It was previously reported that the adrenomedullin and its receptor gene expression, the adrenomedullin concentration and the adrenomedullin binding activity in the heart were also up-regulated in rats with myocardial infarction (Nagaya et al., 2000; Oie et al., 2000). Furthermore, we previously reported that adrenomedullin suppressed mitogenesis and collagen synthesis (Horio et al., 1999), suggesting that adrenomedullin might suppress cardiac remodeling after myocardial infarction. Taken together, the present findings suggest that adrenomedullin may play a role as an autocrine/paracrine modulator in the process of not only cardiac remodeling but also cardiac myocyte death in the pathophysiology of acute myocardial infarction.

In conclusion, hypoxia and oxidative stress induced adrenomedullin secretion from cardiac myocytes. An oxidative stress might be involved, in part, in the increased adrenomedullin secretion under hypoxic conditions. Increased adrenomedullin secretion from cardiac myocytes might play a cell protective role in an autocrine manner.

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References

Aburaya, M., Minamino, N., Hino, J., Kangawa, K., Matsuo, H., 1989. Distribution and molecular forms of brain natriuretic peptide in the central nervous system, heart and peripheral tissue of rat. Biochem. Biophys. Res. Commun. 165, 880–887.

Ando, K., Ito, Y., Kumada, M., Fujita, T., 1998. Oxidative stress increases adrenomedullin mRNA levels in cultured rat vascular smooth muscle cells. Hypertens. Res. 21, 187–191.

Chen, Y.F., Durand, J., Claycomb, W.C., 1997. Hypoxia stimulates atrial natriuretic peptide gene expression in cultured atrial cardiocytes. Hypertension 29, 75–82.

Chun, T.H., Itoh, H., Saito, T., Yamahara, K., Doi, K., Mori, Y., Ogawa, Y., Yamashita, J., Tanaka, T., Inoue, M., Masatsugu, K., Sawada, N., Fukunaga, Y., Nakao, K., 2000. Oxidative stress augments secretion of endothelium-derived relaxing peptides, C-type natriuretic peptide and adrenomedullin. J. Hypertens. 18, 575–580.

Claycomb, W.C., Lanson Jr., N.A., Stallworth, B.S., Egeland, D.B., Delcarpio, J.B., Bahinski, A., Izzo Jr., N.J., 1998. HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. Proc. Natl. Acad. Sci. U.S.A. 95, 2979–2984.

Cook, S.A., Sugden, P.H., Clerk, A., 1999. Regulation of bcl-2 family proteins during development and in response to oxidative stress in cardiac myocytes: association with changes in mitochondrial membrane potential. Circ. Res. 85, 940–949.

Cormier-Regard, S., Nguyen, S.V., Claycomb, W.C., 1998. Adrenomedullin gene expression is developmentally regulated and induced by hypoxia in rat ventricular cardiac myocytes. J. Biol. Chem. 273, 17787–17792.

Duranteau, J., Chandel, N.S., Kulisz, A., Shao, Z., Schumacker, P.T., 1998. Intracellular signaling by reactive oxygen species during hypoxia in cardiomyocytes. J. Biol. Chem. 273, 11619–11624.

Horio, T., Nishikimi, T., Yoshihara, F., Nagaya, N., Matsuo, H., Takishita, S., Kangawa, K., 1998. Production and secretion of adrenomedullin in cultured rat cardiac myocytes and nonmyocytes: stimulation by interleukin-1beta and tumor necrosis factor-alpha. Endocrinology 139, 4576–4580.

Horio, T., Nishikimi, T., Yoshihara, F., Matsuo, H., Takishita, S., Kangawa, K., 1999. Effects of adrenomedullin on cultured rat cardiac myocytes and fibroblasts. Eur. J. Pharmacol. 382, 1–9.

Ishimitsu, T., Kojima, M., Kangawa, K., Hino, J., Matsuoka, H., Kitamura, K., Eto, T., Matsuo, H., 1994. Genomic structure of human adrenomedullin gene. Biochem. Biophys. Res. Commun. 203, 631–639.

Kato, H., Shichiri, M., Marumo, F., Hirata, Y., 1997. Adrenomedullin as an autocrine/paracrine apoptosis survival factor for rat endothelial cells. Endocrinology 138, 2615–2620.

Mansuy, P., Mougenot, N., Ramirez-Gil, J.F., Bonnefont-Rousselot, D., Raillecove, F., Komajda, M., Lechat, P., 2000. Effects of prolonged propranolol treatment on left ventricular remodeling and oxidative stress after myocardial infarction in rats. J. Cardiovasc. Pharmacol. 35, 806–813.

Nagata, D., Hirata, Y., Suzuki, E., Kakoki, M., Hayakawa, H., Goto, A., Ishimitsu, T., Minamino, N., Ono, Y., Kangawa, K., Matsuo, H., Omata, M., 1999. Hypoxia-induced adrenomedullin production in the kidney. Kidney Int. 55, 1259–1267.

Nagaya, N., Nishikimi, T., Yoshihara, F., Horio, T., Morimoto, A., Kangawa, K., 2000. Cardiac adrenomedullin gene expression and peptide accumulation after acute myocardial infarction in rats. Am. J. Physiol.: Regul. Integr. Comp. Physiol. 278, R1019–R1026.

Nakayama, M., Takahashi, K., Murakami, O., Shirato, K., Shibahara, S., 1999. Induction of adrenomedullin by hypoxia in cultured human coronary artery endothelial cells. Peptides 20, 769–772.

Nguyen, S.V., Claycomb, W.C., 1999. Hypoxia regulates the expression of the adrenomedullin and HIF-1 genes in cultured HL-1 cardiomyocytes. Biochem. Biophys. Res. Commun. 265, 382–386.

Nishikimi, T., Horio, T., Sasaki, T., Yoshihara, F., Takishita, S., Miyata, A., Matsuo, H., Kangawa, K., 1997. Cardiac production and secretion of adrenomedullin are increased in heart failure. Hypertension 30, 1369–1375.

Nishikimi, T., Horio, T., Yoshihara, F., Nagaya, N., Matsuo, H., Kangawa, K., 1998. Effect of adrenomedullin on cAMP and cGMP levels in rat cardiac myocytes and nonmyocytes. Eur. J. Pharmacol. 353, 337–344.

Norman, D.A., Yacoub, M.H., Barton, P.J., 1998. Nuclear factor NF-kappa B in myocardium: developmental expression of subunits and activation by interleukin-1 beta in cardiac myocytes in vitro. Cardiovasc. Res. 39, 434–441.

Oie, E., Vinge, L.E., Yndestad, A., Sandberg, C., Grogaard, H.K., Attramadal, H., 2000. Induction of a myocardial adrenomedullin signaling system during ischemic heart failure in rats. Circulation 101, 415–422.

Sata, M., Kakoki, M., Nagata, D., Nishimatsu, H., Suzuki, E., Aoyagi, T., Sugiura, S., Kojima, H., Nagano, T., Kangawa, K., Matsuo, H., Omata, M., Nagai, R., Hirata, Y., 2000. Adrenomedullin and nitric oxide inhibit human endothelial cell apoptosis via a cyclic GMP-independent mechanism. Hypertension 36, 83–88.

Shichiri, M., Kato, H., Doi, M., Marumo, F., Hirata, Y., 1999. Induction of max by adrenomedullin and calcitonin gene-related peptide antagonizes endothelial apoptosis. Mol. Endocrinol. 13, 1353–1363.

Sugo, S., Minamino, N., Kangawa, K., Miyamoto, K., Kitamura, K., Sakata, J., Eto, T., Matsuo, H., 1994. Endothelial cells actively synthesize and secrete adrenomedullin. Biochem. Biophys. Res. Commun. 201, 1160–1166.

Theres, H., Wagner, K.D., Schulz, S., Strube, S., Leiterer, K.P., Romberg, D., Gunther, J., Scholz, H., Baumann, G., Schimke, I., 2000. Oxygen radical system in chronic infarcted rat heart: the effect of combined beta blockade and ACE inhibition. J. Cardiovasc. Pharmacol. 35, 708–715.

Villarreal, F.J., Kim, N.N., Ungab, G.D., Printz, M.P., Dillmann, W.H., 1993. Identification of functional angiotensin II receptors on rat cardiac fibroblasts. Circulation 88, 2849–2861.

Yoshihara, F., Nishikimi, T., Horio, T., Yutani, C., Nagaya, N., Matsuo, H., Ohe, T., Kangawa, K., 2000. Ventricular adrenomedullin concentration is a sensitive biochemical marker for volume and pressure overload in rats. Am. J. Physiol.: Heart Circ. Physiol. 278, H633–H642.