Angiotensin-Converting Enzyme-Independent Angiotensin Formation in a Human Model of Myocardial Ischemia: Modulation of Norepinephrine Release by Angiotensin Type 1 and Angiotensin Type 2 Receptors

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ABSTRACT

Angiotensin II (Ang II) promotes norepinephrine (NE) release from cardiac sympathetic nerve endings. We assessed in a human model in vitro whether locally formed Ang II contributes to NE release in myocardial ischemia. Surgical specimens of human right atrium were incubated in anoxic conditions. After 70 min of anoxia, NE release (carrier-mediated; caused by NE transporter reversal) was 8-fold greater than normoxic release. Angiotensin-converting enzyme inhibition with enalaprilat failed to reduce anoxic NE release. In contrast, prevention of chymase-dependent Ang II formation with chymostatin, Bowman-Birk inhibitor, or α-antitrypsin significantly inhibited anoxic, but not exocytotic, NE release. Two mast-cell stabilizers, cromolyn and lodoxamide, markedly reduced NE release, implicating mast-cell-dependent Ang II formation increases in myocardial ischemia (Jalowy et al., 1999). Locally generated Ang II could, therefore, contribute to NE release associated with myocardial ischemia. Indeed, in a human model in vitro whether locally formed Ang II to ischemic NE release in the human heart. Locally generated Ang II promotes NE release by acting predominantly at AT1Rs, which are likely uncovered between EXP3174 and an AT2R agonist, and between EXP3174 and a Na+/H+ exchanger inhibitor. Thus, angiotensin-converting enzyme-independent Ang II formation via chymase is important for carrier-mediated ischemic NE release in the human heart. Locally generated Ang II promotes NE release by acting predominantly at AT1Rs, which are likely covered by sympathoadrenergic activation. In the ischemic heart, a vicious circle may develop whereby enhanced norepinephrine (NE) release initiates arrhythmias that can further reduce oxygen supply, thus worsening ischemia and associated arrhythmias. The process may culminate in sudden cardiac death (Scho¨mig et al., 1995); therefore, modulation of NE release has important clinical implications.

It has been known for some time that angiotensin II (Ang II) increases the response to sympathetic stimulation in the cutaneous vasculature (Zimmerman and Gomez, 1965). More recently, Ang II was found to be a potent facilitator of NE release from cardiac sympathetic nerve endings (Rump et al., 1994; Seyedi et al., 1997). A renin-angiotensin system is present in the heart (Dostal and Baker, 1999), and Ang II formation increases in myocardial ischemia (Jalowy et al., 1999). Locally formed Ang II could, therefore, contribute to NE release associated with myocardial ischemia. Indeed, in an isolated guinea pig heart model of ischemia/reperfusion, Ang II reduces both NE release and the severity of associated arrhythmias (Maruyama et al., 1999).

The purpose of this study was to assess the contribution of locally formed Ang II to ischemic NE release in the human heart. For this, we used a human model of protracted myocardial ischemia adopted in our laboratory (Hatta et al., 1999). Oxygen demand and arrhythmogenesis are both enhanced by sympathoadrenergic activation. In the ischemic heart, a vicious circle may develop whereby enhanced norepinephrine (NE) release initiates arrhythmias that can further reduce oxygen supply, thus worsening ischemia and associated arrhythmias. This process may culminate in sudden cardiac death (Scho¨mig et al., 1995); therefore, modulation of NE release has important clinical implications.

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creases, secondary to Na\(^+/\text{H}^+\) exchanger activation. This triggers the reversal of the NE transporter and, hence, a massive release of NE (i.e., carrier-mediated NE release) (Levi and Smith, 2000).

In view of the importance of angiotensin-converting enzyme (ACE)-independent pathways in Ang II formation in the human heart (mostly chymase) (Balecels et al., 1997; Wolny et al., 1997; Akasu et al., 1998), we focused our investigation on chymase-generated Ang II and the cellular source of this enzymatic activity. Because myocardial ischemia alters the expression of Ang II-receptor subtypes (Nio et al., 1995; Wharton et al., 1998), we also examined the respective roles of angiotensin type 1 receptor (AT\(_1\)R) and angiotensin type 2 (AT\(_2\)R) in this setting.

We report that, in the ischemic human myocardium, mast cell-derived chymase contributes significantly to Ang II formation and NE release. AT\(_1\)Rs mediate this action of Ang II, whereas AT\(_2\)Rs may have opposite effects.

### Materials and Methods

**Source of Human Cardiac Tissue.** Specimens of right atrium (i.e., surgical waste tissue) were obtained from 72 patients undergoing cardiopulmonary bypass (68 males and 4 females, age 66 ± 1.3 years; coronary artery bypass grafting, 67; valve replacement, 5), following a protocol approved by our Institutional Review Board. Seventeen of the 67 patients who underwent coronary artery bypass grafting were chronically treated with β-adenoreceptor blocking agents. Preoperative treatment with β-blockers did not affect the anoxic release of NE. All patients chronically treated with ACE inhibitors were excluded from the study. At the time of surgery, a piece of atrial appendage measuring ~1 cm\(^2\) was removed from the atriotomy site.

**Incubation Conditions.** The specimen was immediately transported to the laboratory in ice-cold oxygenated Krebs-Henseleit solution (KHS) of the following composition (mM): NaCl, 118.2; KCl, 4.83; CaCl\(_2\), 2.5; MgSO\(_4\), 2.37; KH\(_2\)PO\(_4\), 1.0; NaHCO\(_3\), 25; and glucose, 11.1. After removal of fat and connective tissue, the specimen was divided into several fragments (each weighing 25.8 ± 0.6 mg, wet weight, measured at the end of incubation). Each fragment was incubated for 15 min at 37.5°C in 2 ml of KHS gassed with 95% O\(_2\) and 5% CO\(_2\) and contained the reducing agent sodium dithionite (3 mM; pO\(_2\) 70 mm Hg; pH 7.4) causing a pronounced carrier-mediated release of endogenous NE (Hatta et al., 1997). As shown in Fig. 1, after 70 min of anoxia, NE release was ~800 pmol/g (i.e., ~8-fold greater than normoxic control). The selective bradykinin (BK) type 2 receptor (B\(_2\)R) antagonist Hoe 140 (30 nM; Wirth et al., 1995) and the kininase II/ACE inhibitor enalaprilat (1 μM; K\(_i\), 0.1 nM; Weisser and Schloos, 1991) each failed to affect anoxic NE release (Fig. 1 A and C). In contrast, when Hoe 140 (30 nM) and enalaprilat (1 μM) were used in combination, NE release was attenuated by 25% (Fig. 1D). The AT\(_1\)R antagonist EXP 3174 (100 nM; K\(_i\), 10 nM; Wienen et al., 1992) also caused a marked decrease (~50%) in anoxic NE release (Fig. 1B).

**Results.**

The incubation of human right atrial tissue in glucose-free KHS in anoxic conditions (pO\(_2\) ~0 mm Hg; pH ~7.3) caused a pronounced carrier-mediated release of endogenous NE (Hatta et al., 1997). As shown in Fig. 1, after 70 min of anoxia, NE release was ~800 pmol/g (i.e., ~8-fold greater than normoxic control). The selective bradykinin (BK) type 2 receptor (B\(_2\)R) antagonist Hoe 140 (30 nM; K\(_i\), 0.3 nM; Wirth et al., 1995) and the kininase II/ACE inhibitor enalaprilat (1 μM; K\(_i\), 0.1 nM; Weisser and Schloos, 1991) each failed to affect anoxic NE release (Fig. 1 A and C). In contrast, when Hoe 140 (30 nM) and enalaprilat (1 μM) were used in combination, NE release was attenuated by 25% (Fig. 1D). The AT\(_1\)R antagonist EXP 3174 (100 nM; K\(_i\), 10 nM; Wienen et al., 1992) also caused a marked decrease (~50%) in anoxic NE release (Fig. 1B).

The human chymase inhibitor BBI (10 nM) reduced anoxic NE release by 25% (Fig. 2A), whereas the trypsin inhibitor BPTI (10 μM), which is devoid of antichymase activity (Johnson et al., 1988), was ineffective (Fig. 2B). Two other human chymase inhibitors, chymostatin (100 μM) and α\(_1\)-antiproteinase (1 μM), each reduced anoxic NE release by ~40% (Fig. 2, C and D), but neither affected exocytotic NE release elicited by depolarization with 50 mM K\(^+\) (Fig. 3). In contrast, exocytotic NE release was attenuated in Ca\(^{2+}\)-free medium or by activation of histamine H\(_3\)-receptors (Fig. 3).

Inasmuch as these findings suggested an involvement of chymase in anoxic NE release, we questioned whether mast cells may be a source of this chymase. As shown in Fig. 4, the mast-cell stabilizing agents cromolyn (100 μM; panel A) and
loadoxamide (10 μM; panel B) attenuated anoxic NE release by ~30% and ~45%, respectively.

Inhibition of the chymase pathway with chymostatin (100 μM) resulted in a ~30% decrease in NE release that was abolished by the addition of enalaprilat (1 μM). The further addition of Hoe 140 (30 nM) reinstated the inhibitory effect of chymostatin (Fig. 5). The selective AT1R antagonist EXP 3174 decreased NE release by ~40% Fig. 3. Exocytotic NE release from human right atrium in normoxic conditions. Specimens were incubated for 5 min in normal KHS containing 5.83 mM K+ (basal) or 50 mM K+ (K+, depolarizing solution); in either 2.5 mM Ca2+ or 0 mM Ca2+ plus EGTA 5 mM (0 Ca2+); in the presence or absence of α1-antitrypsin (α1-AT, 1 μM), chymostatin (Chymo, 100 μM), and the histamine H3-receptor agonist imetit (100 nM). Bars (mean ± S.E.; n = 4) represent the total NE released in 5 min. *P < .05 and **P < .01, significantly different from basal and K+-induced release, respectively, by one-way ANOVA with Bonferroni’s t test used for post hoc analysis.

Fig. 4. NE release from human right atrium incubated in anoxic conditions. Specimens were incubated without any drug (anoxia) or with the mast cell stabilizers cromolyn (100 μM; panel A; n = 7) and lodoxamide (10 μM; panel B). Bars (mean ± S.E.) represent the total NE released during 70 min of anoxia. **P < .01 and ***P < .001, significantly different from anoxia by paired Student’s t test.

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When PD 123319 (1 μM) was added to EXP 3174 (100 nM), the effect of EXP 3174 was abolished (Fig. 6). The selective AT2R agonist (p-amino-Phe6)-Ang II (30 nM; Ki, 12 nM; Speth and Kim, 1990) caused a moderate, albeit not statistically significant, attenuation of anoxic NE release (Fig. 7). Notably, when (p-amino-Phe6)-Ang II was added to a subthreshold concentration of EXP 3174 (10 nM), anoxic NE release was significantly reduced by ~60% (Fig. 7).

We had previously reported that the Na+/H+ exchanger inhibitor EIPA (10 μM; Hatta et al., 1997) markedly reduces anoxic NE release in this human model of protracted myocardial ischemia. Shown in Fig. 8 is the finding that whereas subthreshold concentrations of EXP 3174 (10 nM) and EIPA (3 μM) each failed to affect anoxic NE release, they decreased anoxic release by ~40% when used in combination.

**Discussion**

Collectively, our findings indicate that ACE-independent Ang II formation plays an important role in the carrier-mediated release of NE associated with protracted ischemia in the human heart. Locally generated Ang II promotes NE release by acting predominantly at the AT1R. Effects of Ang
II at the AT1R, seemingly opposite to those resulting from AT1R activation, are uncovered when AT1Rs are blocked.

BK facilitates NE release from cardiac sympathetic nerve endings (Seyedi et al., 1997, 1999), and its production increases in myocardial ischemia (Matsuki et al., 1987). Furthermore, B2R stimulation by exogenous BK markedly enhances carrier-mediated NE in a human model of myocardial ischemia (Hatta et al., 1999). We suspected that endogenous BK may contribute to ischemic NE release in the human heart because, when BK formation was inhibited with serine proteinase inhibitors, NE release was decreased, whereas when BK catabolism was prevented by a combination of kininase I and II inhibitors, NE release was enhanced (Hatta et al., 1999). Yet, we now report that the BK B2R antagonist Hoe 140 failed to affect ischemic NE release in the same human model of protracted myocardial ischemia, whereas the Ang II AT1R antagonist losartan markedly inhibited it (Münch et al., 1996). Similarly, the Ang II AT1R antagonist losartan was found by others to inhibit NE release from the anoxic human heart (Münch et al., 1996). This suggests that locally formed Ang II, rather than BK, plays a role in this process. Indeed, endogenously released Ang II fully activates AT1R in this preparation of ischemic human heart, as indicated by the finding that the addition of exogenous Ang II fails to further enhance anoxic NE release, whereas the AT1R antagonist losartan markedly inhibits it (Münch et al., 1996).

Local Ang II formation in the mammalian heart is supported by several findings. First, all of the renin-Ang II system components are synthesized in situ (Dostal and Baker, 1999). Second, administration of Ang I to the human heart in vitro promotes NE exocytosis, and this response is blocked either by ACE inhibitors or AT1R antagonists (Rump et al., 1998). Nevertheless, the possibility remains that Ang II is released in ischemic condition from tissue storage sites because Ang II has been found by immunoelectron microscopy to be present in secretory granule-like structures in cardiomyocytes (Sadoshima et al., 1993).

Although enalaprilat did not reduce ischemic NE release, this does not diminish the role of Ang II that we now advocate. Enalaprilat not only prevents ACE-dependent Ang II formation, but also inhibits BK breakdown by kininase II (Blais et al., 1997). In fact, when the facilitatory role of BK was blocked with Hoe 140, a modest but significant decrease in NE release occurred with enalaprilat (see Fig. 1D).

The finding that Ang II AT1R blockade was more effective than ACE inhibition combined with BK B2R blockade suggested that the Ang II involved in ischemic NE release may derive primarily from an ACE-independent pathway. Indeed, Ang II can be formed in the heart by serine proteases (Arakawa, 1996), and serine proteinase inhibitors attenuate NE release in a human model of myocardial ischemia (Hatta et al., 1999). This prompted us to assess the role of Ang II formed via the ACE-independent pathway (Dostal and Baker, 1999) in the release of NE in myocardial ischemia.

Because chymase, a chymotrypsin-like serine proteinase, appears to be important in the ACE-independent generation of Ang II from Ang I in the heart (Akaue et al., 1998), we hypothesized that this enzymatic pathway may play a role in the Ang II-induced facilitation of ischemic NE release. Indeed, each of three inhibitors of human chymase, BBI (Ware et al., 1997), α1-antitrypsin (Kokkonen et al., 1997), and chymostatin (Urata et al., 1990b), effectively reduced anoxic NE release. In contrast, the trypsin inhibitor BPTI, which is devoid of antichymase activity (Johnson et al., 1988), was ineffective. Notably, both α1-antitrypsin and chymostatin failed to affect exocytic NE release, which was instead inhibited by Ca2+ removal or by stimulation of pre-synaptic histamine H3-receptors (see Fig. 3) (Levi and Smith, 2000). The fact that chymase inhibitors selectively inhibit carrier-mediated NE release indicates that chymase-formed Ang II plays an important role in the release of NE associated with protracted myocardial ischemia in humans.

Kokkonen et al. (1997) suggested that interstitial fluid in the human heart contains natural chymase inhibitors that would prevent chymase-dependent Ang II formation in vivo. Inasmuch as natural chymase inhibitors may play a lesser role in our in vitro preparation, given the paucity of interstitial fluid, it may be difficult to extrapolate our data to the intact heart. However, recent evidence from another laboratory (Takai et al., 1999) indicates that chymase is released from mast cells in a heparin-bound form, which would make it resistant to natural chymase inhibitors. Thus, whether interstitial fluid is present or not, our findings may still be relevant to human pathophysiology.

Human heart mast cells display high chymase activity (Sperr et al., 1994; Patella et al., 1995), and two mast cell stabilizers, cromolyn (Parikh and Singh, 1998) and lodoxamide (Jolly et al., 1982; Keller et al., 1988), markedly reduced NE release in our study (see Fig. 4). It is therefore likely that mast cells function as a major source of the chymase-generated Ang II, which promotes ischemic NE release in the human heart. Release of tryptase and chymase from mast cells contributes to atherosclerotic plaque rupture and, hence, coronary artery occlusion (Kaartinen et al., 1994). Moreover, mast cell chymase induces apoptosis in cardiomyocytes, while increasing the proliferation of nonmyocardial cells, thus contributing to the progression of heart failure (Hara et al., 1999). Accordingly, mast cell stabilizers may offer further advantages, in addition to reducing ischemic NE release.

Although cromolyn and lodoxamide inhibit histamine release from mast cells (Theoharides et al., 1980; Mackay and Pearce, 1996; Van Haaster et al., 1996), it is unlikely that this action would result in an attenuation of ischemic NE release. In fact, although histamine release is augmented in this human model of myocardial ischemia, it negatively modulates NE release by activating histamine H4-receptors (Hatta et al., 1997). If at all, therefore, cromolyn and lodoxamide would be expected to enhance, rather than attenuate, NE release.

The addition of enalaprilat reversed the inhibition of NE release by chymostatin; however, the further addition of a BK B2R antagonist reinstated this effect (see Fig. 5). This suggests that by prolonging the half-life of BK (Hatta et al., 1999), enalaprilat potentiated the NE-releasing effect of endogenous BK and thus counteracted the inhibitory effect of chymostatin.

Notably, when both ACE-dependent and -independent pathways of Ang II formation were blocked and the effects of BK were also antagonized, the inhibition of NE release was not greater than when only the ACE-independent pathway was interrupted (see Fig. 5). It is possible that when ACE and chymase are both inhibited, NE release is promoted by another factor, probably angiotensin-(1-7). Indeed, angiotensin-
(1-7) facilitates the release of NE in the rat heart (Gironacci et al., 1994) and can be formed from Ang I by neutral endopeptidase (Yamamoto et al., 1992), whose activity is present in the human heart (Kokkonen et al., 1999).

The data obtained with the selective Ang II receptor subtype antagonists indicate that AT1Rs, but not AT2Rs, mediate the promotion of ischemic NE release by Ang II. Interestingly, when the activation of AT1Rs was prevented by EXP 3174 and ischemic NE release was thus attenuated, the addition of an AT2R antagonist reversed the effect of the AT1R antagonist (see Fig. 6). Similarly, the reduction of infarct size afforded by AT1R blockade is lost with the concomitant inhibition of AT2R (Jalowy et al., 1998). It is plausible that the primary action of Ang II in our model is to promote ischemic NE release via AT1R activation. Once AT1Rs are blocked, Ang II could inhibit NE release by activating AT2Rs. Although the selective AT2R agonist [p-amino-Phe6]-Ang II failed to further decrease NE release in the presence of EXP 3174 (100 nM; data not shown), [p-amino-Phe6]-Ang II significantly attenuated anoxic NE release when combined with a lower concentration of EXP 3174 (10 nM; see Fig. 7). The higher concentration of EXP 3174 may produce a maximal inhibitory effect on anoxic NE release, thus explaining the lack of effect by [p-amino-Phe6]-Ang II when combined with a higher concentration of EXP 3174. Likewise, AT1R activation was found to facilitate the hypertensive response caused by partial AT1R blockade in hypertensive rats (Barber et al., 1999). Overexpression of AT1R (Masaki et al., 1998) or, conversely, targeted deletion of the AT1R gene (Siragy et al., 1999), further demonstrates that AT1Rs play a counter-regulatory protective role against the actions mediated by AT1Rs. This collective evidence favors a dual function of locally formed Ang II in ischemic NE release, both facilitatory and inhibitory, mediated by AT1Rs and AT2Rs, respectively.

In protracted myocardial ischemia, free NE accumulates in the axoplasm of adrenergic terminals due to diminished vesicular storage, whereas intraneuronal Na+ increases, secondary to Na+/H+ exchanger activation. This triggers the reversal of the NE transporter and, hence, a massive release of NE (i.e., carrier-mediated NE release) (Levi and Smith, 2000). Ang II is known to activate the Na+/H+ exchanger (Gunasegaram et al., 1999). This action could play a significant role in the promotion of NE release by Ang II in protracted myocardial ischemia, as suggested by the synergism between EXP 3174 and EIPA (see Fig. 8).

In conclusion (see Fig. 9), in protracted human myocardial ischemia, Ang II is formed locally from Ang I predominantly by mast cell-derived chymase. Ang II promotes NE release by acting at AT1Rs on sympathetic nerve terminals (Foucart et al., 1996; Seyed et al., 1997), probably by activating the Na+/H+ exchanger, a key signal for initiating carrier-mediated NE release. Although the AT1R-mediated enhancement of NE release is likely to prevail, Ang II may also exert an AT2R-mediated inhibitory effect, which is unmasked when AT1Rs are blocked. Despite the increased BK production in myocardial ischemia (Matsuki et al., 1987), the contribution of BK to ischemic NE release (Hatta et al., 1999) is probably less important than that of Ang II (Maruyama et al., 1999). Indeed, BK B2R blockade fails to modify ischemic NE release, and the effects of BK are only seen when BK degradation is prevented or when chymase-induced Ang II formation is inhibited.

Sympathoadrenergic activation increases oxygen demand and leads to severe arrhythmias, thus exacerbating myocardial ischemia (Schömg et al., 1995). Mast cell density and chymase content are increased in the ischemic heart (Patella et al., 1998) and hypercholesterolemia up-regulates AT1Rs (Nickenig et al., 1997). Accordingly, the notion that chymase-generated Ang II plays a major role in carrier-mediated NE release may have important clinical implications. Indeed, alternative pathways of Ang II formation have been shown to restore tissue levels of Ang II despite ACE inhibition (Urata et al., 1990a; Balcells et al., 1997). Furthermore, AT1R blockade unmasks a likely beneficial effect of AT2R activation. Hence, AT1R antagonists may be preferable to ACE inhibitors in myocardial ischemia, as suggested by a lower mortality rate and a trend to lower plasma NE levels, in patients treated with losartan rather than with captopril (ELITE 1 study) (Pitt et al., 1997).

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References


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