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Norepinephrine Release from the Ischemic Heart Is Greatly Enhanced in Mice Lacking Histamine H_3 Receptors

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ABSTRACT

We previously reported that histamine H_3 receptors (H_3Rs) are present in cardiac sympathetic nerve endings (cSNE) of animals and humans, where they attenuate norepinephrine (NE) release in normal and hyperadrenergic states, such as myocardial ischemia. The recent creation of a transgenic line of mice lacking H_3R provided us with the opportunity to assess the relevance of H_3R in the ischemic heart. We isolated SNE from hearts of wild-type ($H_3R^{+/+}$) and knockout ($H_3R^{-/-}$) mice and found that basal NE release from $H_3R^{-/-}$ cSNE was ~60% greater than that from $H_3R^{+/+}$ cSNE. NE exocytosis evoked by K^+-induced depolarization of cSNE from $H_3R^{+/+}$ mice was attenuated by activation of either H_3R or adenosine A_1 receptors (A_1R). In contrast, NE release from cSNE of $H_3R^{-/-}$ was unaffected by H_3R agonists, but it was still attenuated by A_1R activation. When isolated mouse hearts were subjected to ischemia for 20 min, NE overflow into the coronaries was 2-fold greater in the $H_3R^{-/-}$ hearts than in those from $H_3R^{+/+}$ mice. Furthermore, whereas stimulation of H_3R or A_1R reduced ischemic NE overflow from $H_3R^{+/+}$ hearts by 50%, only A_1R, but not H_3R activation, reduced NE release in $H_3R^{-/-}$. Our data demonstrate that NE release from cSNE can be modulated by various heteroinhibitory receptors (e.g., H_3R and A_1R) and that H_3Rs are particularly important in modulating NE release in myocardial ischemia. Inasmuch as excessive NE release is clinically recognized as a major cause of arrhythmic cardiac dysfunction, our findings reveal a significant cardioprotective role of H_3R on cSNE.

Sympathetic overactivity accompanied by excessive norepinephrine (NE) release is clinically recognized as a major cause of arrhythmic cardiac dysfunction in myocardial ischemia (Braunwald and Sobel, 1988; Kurz et al., 1991; Dart and Du, 1993; Kubler and Strasser, 1994; Benedict et al., 1996). Indeed, myocardial infarction is often accompanied by arrhythmias with high morbidity and mortality (Braunwald and Sobel, 1988; Schomig et al., 1995; Airaksinen, 1999). Sympathetic overactivity and excessive NE release increase metabolic demand, thereby aggravating the primary ischemia and initiating a vicious cycle that can culminate in further myocardial damage and severe cardiac failure (Kubler and Strasser, 1994). Moreover, once released, NE enhances intracellular Ca^{2+} by increasing its influx through voltage-dependent channels, mobilizing it from intracellular stores and favoring its inward transport by the Na^+/Ca^{2+} exchanger. Ca^{2+} overload eventually results in dysrhythmia and uncoordinated myocyte contraction (Levi and Smith, 2000). Therefore, negative modulation of NE release from cardiac sympathetic nerves is a crucial protective mechanism.

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We have shown that activation of histamine H_3 receptors (H_3Rs) on cardiac sympathetic nerve endings (cSNE) negatively modulates NE release from ischemic hearts and attenuates the severity of associated ventricular arrhythmias (Levi and Smith, 2000). H_3Rs are but one of several classes of prejunctional heteroinhibitory receptors (Imamura et al., 1996), and their efficacy in myocardial ischemia models has been tested to date only by pharmacological antagonism of their effects (Levi and Smith, 2000). The availability of a newly created transgenic line of mice lacking H_3R (Toyota et al., 2002) permits us to compare myocardial ischemia in the absence and presence of H_3R and, thus, to evaluate the relevance of H_3R as a basic modulatory mechanism of ischemic NE release. We report the novel finding that hearts with H_3R deletion release more than twice as much NE when subjected to ischemia than hearts with intact H_3R. This finding underscores the relevance of H_3R as a major cardioprotective mechanism in myocardial ischemia.

Materials and Methods

Generation of Histamine $H_3R^{-/-}$ Mice. $H_3R^{-/-}$ knockout mice were generated, and deletion was verified with radioligand binding

ABBREVIATIONS: NE, norepinephrine; H_3R, histamine H_3 receptor; A_1R, adenosine A_1 receptor; cSNE, cardiac sympathetic nerve endings; ANOVA, analysis of variance; KHB, Krebs-Henseleit buffer; SNE, sympathetic nerve endings; CPA, N^6-cyclopentyladenosine; DPCPX, 3-cyclopentyl-1,3-dipropylxanthine.

and pharmacological challenge as described previously (Toyota et al., 2002).

NE Release from Ischemic Mouse Hearts. Male wild-type $H_3R^{+/+}$ (body weight, 26.6 ± 0.4 g; heart weight, 141 ± 3 mg; $n = 49$) and knockout $H_3R^{-/-}$ mice (body weight, 27.2 ± 0.4 g; heart weight, 144 ± 2 mg; $n = 35$) were killed by cervical dislocation under light anesthesia with CO_2 vapor in accordance with institutional guidelines. The ribcage was dissected away, and the heart was rapidly excised, freed from fat and connective tissue and transferred to a Langendorff apparatus. The aorta was cannulated with a flanged 18-gauge stainless-steel needle. Spontaneously beating hearts were perfused through the aorta in a retrograde mode at a constant pressure of 100 cm of H_2O with modified Krebs-Henseleit buffer (KHB) containing 120 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 11 mM glucose, and 0.5 mM EDTA. The perfusion fluid was equilibrated with 95% O_2/5% CO_2 at 37°C to give a pH of 7.4. After a 30-min stabilization period, normothermic ischemia was induced by perfusing hearts for 20 min with glucose-free KHB equilibrated with 95% N_2 and 5% CO_2 and containing the reducing agent sodium dithionite (final concentration of 0.25 mM). Hearts receiving drug treatment were treated for 15 min before induction of ischemia. The coronary effluent was collected into tubes. In the preischemic and ischemic periods, tubes were replaced every 5 min. The volume of effluent collected for each period was weighed and subsequently analyzed for NE content. All drugs were added to the perfusion solution. NE was assayed in the coronary perfusate by high-pressure liquid chromatography with electrochemical detection (Silver et al., 2002).

NE Release from Cardiac Synaptosomes. Cardiac synaptosomes were isolated as described previously for the guinea pig (Seyedi et al., 1997; Silver et al., 2002). Briefly, hearts from 20 $H_3R^{+/+}$ and 20 $H_3R^{-/-}$ mice were excised as described above and transferred to a Langendorff apparatus. Spontaneously beating hearts were perfused through the aorta for 15 min at constant pressure (100 cm of H_2O) with modified KHB at 37°C saturated with 95% O_2 and 5% CO_2, pH 7.4. This procedure ensured that no blood traces remained in the coronary circulation. At the end of the 15-min perfusion, hearts were minced in ice-cold 0.32 M sucrose containing 1 mM EGTA, pH 7.4. Minced tissue was digested with 40 mg collagenase (type II; Worthington Biochemicals, Freehold, NJ) per 10 ml of 0.32 M sucrose solution per gram of wet heart weight for 1 h at 37°C. The sucrose solution contained 1 mM pargyline to prevent enzymatic destruction of synaptosomal NE. After low-speed centrifugation (10 min at 120*g* and 4°C), the resulting pellet was suspended in 10 volumes of 0.32 M sucrose and homogenized with a Teflon/glass homogenizer. The homogenate was spun at 650*g* for 10 min at 4°C, and the pellet was rehomogenized and respun. The pellet containing cellular debris was discarded, and the supernatants from the last two spins were combined and equally subdivided into four tubes. Each tube was centrifuged for 20 min at 20,000*g* at 4°C. This pellet, which contained cardiac synaptosomes, was resuspended in Hepes-buffered saline to a final volume of 500 μl in the presence or absence of pharmacological agents for a total of 20 min in a water bath at 37°C. Each suspension functioned as an independent sample and was used only once. In every experiment, one sample was untreated (control, basal NE release), and others were incubated with drugs for 20 min. When antagonists were used, samples were incubated with the antagonist for 20 min before incubation with the agonist. Controls were incubated for an equivalent length of time without drugs. At the end of the incubation period, each sample was centrifuged for 20 min (20,000*g* at 4°C). The supernatant was assayed for NE content by high-pressure liquid chromatography as described above, and the pellet was assayed for protein content by a modified Lowry procedure (Silver et al., 2002). Although the presence of sympathetic nerve endings in the synaptosomal preparation was not verified by electron microscopy, murine cardiac synaptosomes responded to K^+ depolarization with NE release, which was inhibited by selective H_3R and A_1R activation (as described under *Results*). This response was indistinguishable from that observed in the same preparation from the guinea pig heart (Seyedi et al., 1997), whose synaptosomal composition had been ascertained by electron microscopy (R. Levi and N. Seyedi, unpublished observations).

Statistics. Values are expressed as the mean percentage increases above basal NE release (synaptosomes) or as absolute values for NE overflow (isolated hearts) ± S.E.M. Analysis by one-way ANOVA was used, followed by post hoc testing (Dunnett's test). A p value of <0.05 was considered statistically significant.

Results

Exocytosis of Endogenous Norepinephrine from Cardiac Sympathetic Nerve Terminals. As we did previously with human (Imamura et al., 1995), guinea pig (Seyedi et al., 1997), and dog (Seyedi et al., 1996) cardiac tissue, we first assessed whether the mouse heart harbors H_3 inhibitory heteroreceptors located prejunctionally on SNE. For this, we studied the action of the selective H_3R agonist imetit (Garbarg et al., 1992) directly on SNE (cardiac synaptosomes) isolated from wild-type ($H_3R^{+/+}$) mouse hearts. As shown in Fig. 1, A and B, depolarization of mouse cSNE with 100 mM K^+ resulted in a ~20 to 30% increase in NE release above the basal level of 0.76 ± 0.11 pmol/mg (mean ± S.E.M.; $n = 20$). When cSNE were pretreated with imetit (100 nM), NE release in response to K^+-induced depolarization was reduced by ~50%. This effect of imetit was prevented by pretreatment with the selective H_3R antagonist thioperamide (Arrang et al., 1987) (300 nM) (Fig. 1A). We also determined the presence of other prejunctional inhibitory heteroreceptors in the cSNE of $H_3R^{+/+}$ mice. As shown in Fig. 1B, the selective adenosine A_1 receptor (A_1R) agonist N^6-cyclopentyladenosine (CPA; 300 nM) (Barrett et al., 1994) decreased K^+-induced NE release by ~70%. This effect of CPA was prevented by pretreatment with the selective A_1R antagonist 3-cyclopentyl-1,3-dipropylxanthine (DPCPX) (300 nM) (Haleen et al., 1987) (Fig. 1B).

As shown in Fig. 1, C and D, K^+-induced depolarization of cSNE isolated from hearts of mice lacking H_3R ($H_3R^{-/-}$) resulted in a ~25 to 35% increase in NE release above the basal level of 1.25 ± 0.06 pmol/mg (mean ± S.E.M.; $n = 20$). Notably, this basal level was ~60% greater than that for synaptosomes isolated from $H_3R^{+/+}$ mouse hearts ($p < 0.01$). Contrary to its action on SNE from $H_3R^{+/+}$ mouse hearts, imetit failed to modify the K^+-induced NE release in SNE isolated from $H_3R^{-/-}$ mouse hearts (Fig. 1C). However, in $H_3R^{-/-}$ cSNE, activation of A_1R with CPA still caused a ~70% reduction in K^+-induced NE release, which was prevented by pretreatment with DPCPX (Fig. 1D). This suggested that although H_3R-mediated modulation of NE exocytosis had been deleted in $H_3R^{-/-}$ mouse hearts, A_1R-mediated modulation was preserved.

Release of Endogenous Norepinephrine from the Ischemic Heart. Inasmuch as these findings indicated the absence of inhibitory H_3R on cSNE of $H_3R^{-/-}$ mice, we next questioned whether such an absence might influence NE release in myocardial ischemia, given that H_3R are known to negatively modulate NE release in this condition (Levi and Smith, 2000). When hearts from either $H_3R^{+/+}$ or $H_3R^{-/-}$ mice were excised and perfused in a Langendorff apparatus in normoxic conditions, NE overflow into the coronary effluent was below the detection threshold (data not shown). When hearts from $H_3R^{+/+}$ mice were perfused for 20 min in

ischemic conditions (glucose-free buffer containing the reducing agent sodium dithionite and equilibrated with 95% N_2 and 5% CO_2), total NE overflow increased to ~400 pmol/g (Fig. 2A). The NE transporter inhibitor desipramine (100 nM) markedly inhibited (~50%) this increase in overflow (Fig. 2A), indicating that ischemic NE release was carrier-mediated; that is, NE was carried out of cSNE by the NE transporter in a reversed mode of action (Levi and Smith, 2000). In hearts perfused with imetit (100 nM), ischemic NE overflow was reduced by ~40%. This effect was abolished in the presence of thioperamide (300 nM). In fact, with thioperamide, either alone or combined with imetit, ischemic NE overflow was ~35% greater than that in control conditions (Fig. 2A). In hearts perfused with CPA (100 nM), ischemic NE overflow was reduced by ~50%. This effect was abolished in the presence of DPCPX (100 nM).

In marked contrast, when hearts from $H_3R^{-/-}$ mice were perfused for 20 min in ischemic conditions, total NE overflow was more than 2-fold greater than in $H_3R^{+/+}$ mouse hearts ($p < 0.01$) (Fig. 2B). As in $H_3R^{+/+}$ hearts, desipramine (100 nM) markedly inhibited (~65%) this increase in overflow (Fig. 2B), indicating that ischemic NE release in $H_3R^{-/-}$ hearts was also carrier-mediated. However, neither imetit nor thioperamide modified ischemic NE overflow in $H_3R^{-/-}$ hearts (Fig. 2B). Similar to its action on $H_3R^{+/+}$ hearts, CPA (100 nM) again reduced ischemic NE overflow by ~50%, an effect that was prevented by DPCPX (100 nM) (Fig. 2B).

Discussion

In protracted myocardial ischemia, metabolic acidosis develops in SNE, leading to activation of the Na^+/H^+ exchanger and, thus, to an increase in intraneuronal Na^+ concentra-

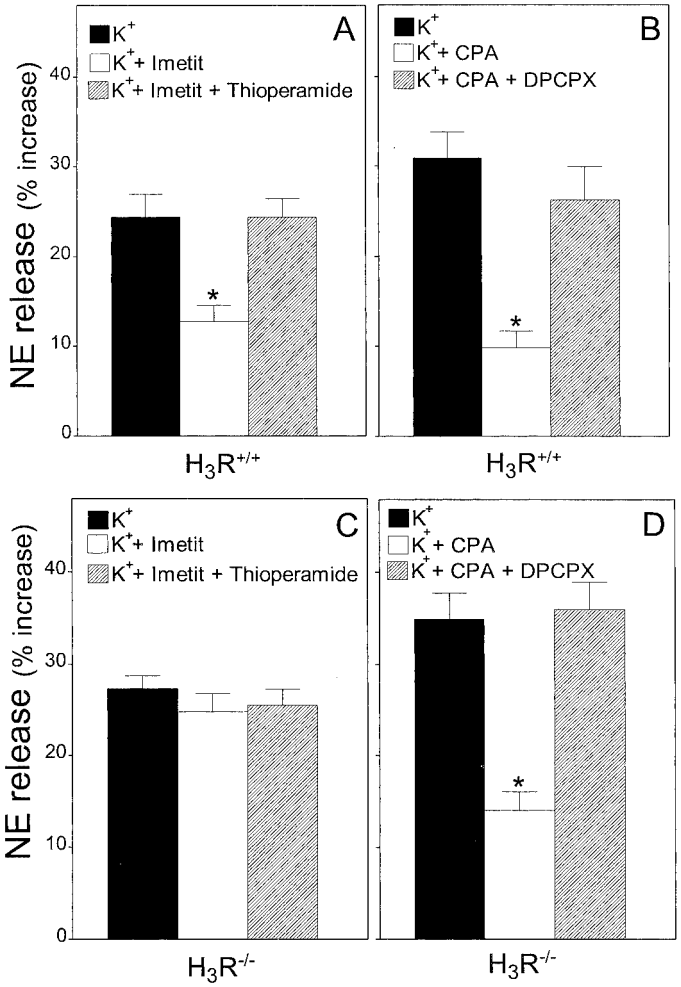

Fig. 1. Exocytotic NE release from mouse heart sympathetic nerve endings (cardiac synaptosomes) depolarized with 100 mM K^+. Bars indicate the mean percentage increases in NE release above basal levels (± S.E.M.). Basal NE release was 0.76 ± 0.11 and 1.25 ± 0.06 pmol/mg protein for $H_3R^{+/+}$ and $H_3R^{-/-}$, respectively ($n = 20 + 20$; $p < 0.01$). Drugs were administered at the following concentrations: imetit, 100 nM; thioperamide, 300 nM; CPA, 300 nM; and DPCPX, 300 nM. These data show that the H_3R-mediated attenuation of NE exocytosis is lost in the synaptosomes of $H_3R^{-/-}$ mice, whereas the modulatory activity of the adenosine A_1R-agonist CPA is preserved. A total of 40 mouse hearts were used ($n = 8$–12 for each graph). *, significantly different from K^+ alone by one-way ANOVA, followed by post hoc testing (Dunnett's test).

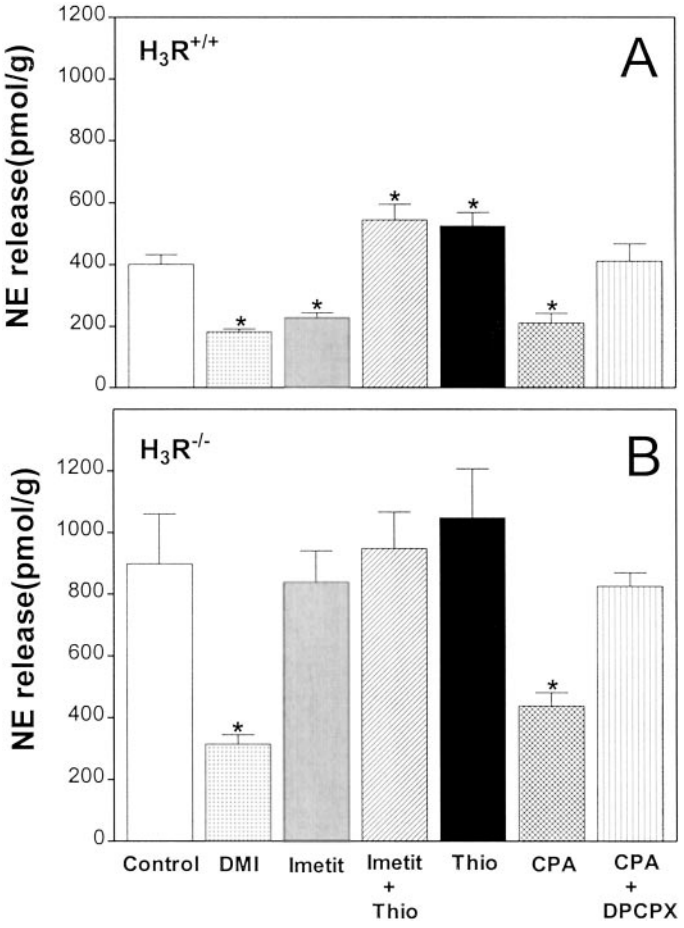

Fig. 2. Carrier-mediated NE release from isolated ischemic mouse hearts. After a stabilization period, each of the 84 hearts was made globally ischemic by a 20-min perfusion with glucose-free KHS containing sodium dithionite 0.25 mM and bubbled with 95% N_2 plus 5% CO_2. Bars are means ± S.E.M. ($n = 5$–11 per column). Control NE release represents the total amount of NE released in the 20-min ischemic period. This release was carrier-mediated because it was inhibited by the NE transporter blocker desipramine (DMI). Drugs were administered at the following concentrations: 100 nM DMI, 100 nM imetit, 300 nM thioperamide, 100 nM CPA, and 100 nM DPCPX. The data show that when subjected to ischemia, $H_3R^{-/-}$ hearts release a 2-fold greater amount of NE than do $H_3R^{+/+}$ hearts ($p < 0.01$), despite the fact the adenosine-mediated modulatory system seems to be functioning as well in the $H_3R^{-/-}$ as in the $H_3R^{+/+}$ hearts. Note that the doubling of ischemic NE release in the $H_3R^{-/-}$ hearts persists in the presence of imetit and thioperamide. *, significantly different from control by one-way ANOVA, followed by post hoc testing (Dunnett's test).

tion. Also, because of ATP depletion and impaired NE storage in synaptic vesicles, NE accumulates in the axoplasm. These conditions force the reversal of the Na^+-dependent NE transporter in an outward direction, triggering a massive carrier-mediated release of NE and arrhythmias (Lameris et al., 2000; Levi and Smith, 2000; Akiyama and Yamazaki, 2001). Indeed, NE overflow in myocardial ischemia directly correlates with the severity of arrhythmias (Imamura et al., 1996; Hatta et al., 1999; Maruyama et al., 1999).

We had identified H_3R as inhibitory heteroreceptors in adrenergic nerve endings of the heart (Endou et al., 1994). We also established that in addition to inhibiting NE exocytosis from sympathetic nerve endings, selective H_3R agonists attenuate carrier-mediated release of NE in both animal and human models of protracted myocardial ischemia (Imamura et al., 1996; Hatta et al., 1997). We subsequently demonstrated that H_3R-mediated attenuation of exocytotic NE release involves an inhibition of N-type Ca^{2+} channels (Silver et al., 2002), whereas H_3R-mediated reduction of carrier-mediated NE release is associated with diminished Na^+/H^+ exchanger activity (Imamura et al., 1996; Hatta et al., 1997; Silver et al., 2001). Most important, by reducing ischemic NE release, H_3R stimulation significantly attenuates the severity of ischemic arrhythmias (Imamura et al., 1996; Levi and Smith, 2000).

Other presynaptic receptors, such as α_2 adrenoceptors and A_1R, also modulate NE release from cSNE (Seyedi et al., 1997). Yet, H_3R stimulation attenuates both exocytotic and carrier-mediated NE release, whereas α_2-adrenoceptor agonists attenuate NE exocytosis but enhance carrier-mediated NE release (Imamura et al., 1996). Furthermore, although A_1R activation reduces both exocytotic and carrier-mediated NE release, A_1R stimulation has negative chronotropic and dromotropic effects, whereas H_3R agonists have no such effects (Levi and Smith, 2000). Accordingly, because excess NE release can trigger severe arrhythmias and sudden cardiac death, we have proposed that negative modulation of NE release by H_3R agonists may offer a novel therapeutic approach to myocardial ischemia (Levi and Smith, 2000; Mackins and Levi, 2000).

The recent creation of a transgenic line of mice devoid of H_3R (Toyota et al., 2002) provided us with the opportunity to assess the relevance of H_3R in myocardial ischemia. Thus, we found that although cSNE isolated from wild-type mice responded to the H_3R agonist imetit with a marked decrease in K^+-induced NE release, similar to what we had observed previously in SNE isolated from guinea pig, dog, and human hearts (Endou et al., 1994; Imamura et al., 1994, 1995; Seyedi et al., 1996; Hatta et al., 1997), cSNE isolated from $H_3R^{-/-}$ mice failed to respond to H_3R agonists with an attenuation of NE exocytosis. Yet $H_3R^{-/-}$ cSNE still responded to A_1R agonists, as demonstrated by the fact that CPA attenuated equally effectively NE exocytosis in cSNE of $H_3R^{+/+}$ and $H_3R^{-/-}$ mice. These findings clearly indicate that $H_3R^{-/-}$ mice are an ideal model for the verification of the postulated cardioprotective role of H_3R located on cSNE.

Indeed, we found that in ischemic conditions, a lack of H_3R in cSNE translated into a 2-fold increase in NE overflow from the hearts of $H_3R^{-/-}$ mice compared with $H_3R^{+/+}$ hearts. This is consistent with our previous findings in the guinea pig heart, in which the blockade of H_3R with thioperamide doubled NE release during ischemia/reperfusion (Imamura et al., 1994), and in a human model of myocardial ischemia, in which blockade of H_3R with thioperamide or clobenpropit significantly increased NE release (Hatta et al., 1997). The massive NE overflow from $H_3R^{-/-}$ mouse hearts occurred despite the fact that inhibitory A_1Rs were still functioning to attenuate both exocytotic and carrier-mediated NE release in the $H_3R^{-/-}$ hearts. This clearly demonstrates that cSNE H_3Rs play a relevant role in the modulation of NE release in myocardial ischemia.

Notably, the H_3R antagonist thioperamide potentiated NE release from ischemic $H_3R^{+/+}$ hearts but not from cSNE from normoxic $H_3R^{+/+}$ hearts. This indicates that, as we had observed previously in guinea pig and human hearts, H_3Rs located on cSNE become activated in conditions characterized by enhanced adrenergic activity, such as myocardial ischemia, when cSNE are exposed to functionally significant concentrations of histamine released from local mast cells by oxygen free radicals (Imamura et al., 1994; Hatta et al., 1997). The fact that thioperamide failed to potentiate NE overflow from ischemic $H_3R^{-/-}$ hearts further strengthens this notion. Basal NE release from cSNE isolated from $H_3R^{-/-}$ was ~60% greater than that from cSNE isolated from $H_3R^{+/+}$ hearts. This finding is consistent with a recent report of constitutive activity of native H_3R in rodent brain (Morisset et al., 2000).

Inasmuch as excessive NE release is recognized as a major cause of arrhythmic cardiac dysfunction in humans (Braunwald and Sobel, 1988; Dart and Du, 1993; Kubler and Strasser, 1994; Benedict et al., 1996), our present and past findings reveal that H_3R perform a crucial protective role in myocardial ischemia. This adds further strength to our notion (Levi and Smith, 2000; Mackins and Levi, 2000) that negative modulation of NE release by H_3R agonists may offer a novel therapeutic approach to myocardial ischemia.

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