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Nitric Oxide and Hydroperoxide Affect Islet Hormone Release and Ca^{2+} Efflux

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We have investigated the influence of the intracellular free radical donors hydroxylamine (giving nitric oxide [NO]) and *tert*-butylhydroperoxide (giving hydroperoxide ["H_2O_2"]) on glucose- and cyclic adenosine monophosphate (cAMP)-induced transduction signaling in islet hormone release. Both donors dose dependently inhibited glucose-stimulated insulin release and induced modest (hydroxylamine) or profound (*tert*-butylhydroperoxide) suppression of $^{45}Ca^{2+}$-efflux from perifused islets. By contrast, both donors stimulated glucagon release. Similar effects on hormone release were displayed after K^+-depolarization. Insulin and glucagon release stimulated by activation of the cAMP system through isobutylmethylxanthine (IBMX) at basal glucose was modestly potentiated by low concentrations of both donors. These effects were still observed, although less pronounced, in K^+-depolarized islets. In vitro as well as in vivo, the NO-synthase inhibitor N^G-nitro-L-arginine methyl ester inhibited IBMX-induced glucagon release, but did not affect insulin release. The results suggest that NO and hydroperoxide inhibit glucose-stimulated insulin release by perturbing Ca^{2+} fluxes and probably acting through *S*-nitrosylation (NO) or oxidation (hydroperoxide) of thiol groups critical to the secretory process. These effects are largely independent of depolarization events. By contrast, both NO and hydroperoxide can potentiate cAMP-stimulated hormone release presumably at a distal site in the stimulus-secretion coupling.

Keywords: Insulin and glucagon secretion; glucose, $^{45}Ca^{2+}$-efflux; cAMP activation; hydroxylamine, *tert*-butylhydroperoxide

Introduction

Nitric oxide (NO) is now known to act as a signal transducer in a variety of mammalian cell types *(1)*. It is an unusual messenger in that it is a free-radical gas and thus conveys biological information in a way greatly different from that of classical transmitters. NO is produced by the enzyme nitric oxide synthase (NOS) *(1)*. There are two major types of constitutive NOS enzymes known to be present in either neurons (nNOS) or endothelial cells (eNOS) *(1,2)*. In brain tissue, NO, derived from nNOS, is not released at the synaptic level, but diffuses from the cell body not to act on conventional receptors but through direct effects on various regulatory processes within the cell or, alternatively, in the cell membrane *(1,2)*. The neuronal isoform of NOS (nNOS) has been detected in endocrine cells in the islets of Langerhans in both the insulin-producing β-cells and the glucagon producing α-cells *(3–6)*. Since NO has been found to have a modulatory influence on both insulin and glucagon release, we have suggested that NO produced from islet nNOS activity might exert its action both within its cell of origin and as a paracrine messenger *(3,6,7–14)*. So far our previous data have indicated that NO has an inhibitory action on glucose-induced insulin release, an action probably exerted through *S*-nitrosylation of certain thiol groups essential for the secretory process *(3,6,9–12,15)*. Similarly, we have previously proposed that an increased production of another free radical, hydrogen peroxide (H_2O_2), induced by stimulation of islet monoamine oxidase (MAO) activity, might negatively modulate glucose-induced insulin release by impairing the function of critical thiol groups *(7,16–19)*. In addition, we have obtained evidence for a stimulating effect by both NO and H_2O_2 on glucagon release *(6,9–14)*. Hence, our previous studies have suggested that islet production of physiological levels of NO and H_2O_2 may serve as important modulators of islet hormone release.

The aim of the present investigation was to study in more detail the effects of NO as compared to that of H_2O_2 on the transduction mechanisms for both insulin and glucagon secretion by using donor compounds that can directly release these free radicals. Since there is no evidence, so far, of a correlation between the effects of endogenous vs exogenous free radicals, we have chosen to test donors that enter cells and release the free radicals intracellularly. Thus, we have used the intracellular NO donor hydroxylamine, a

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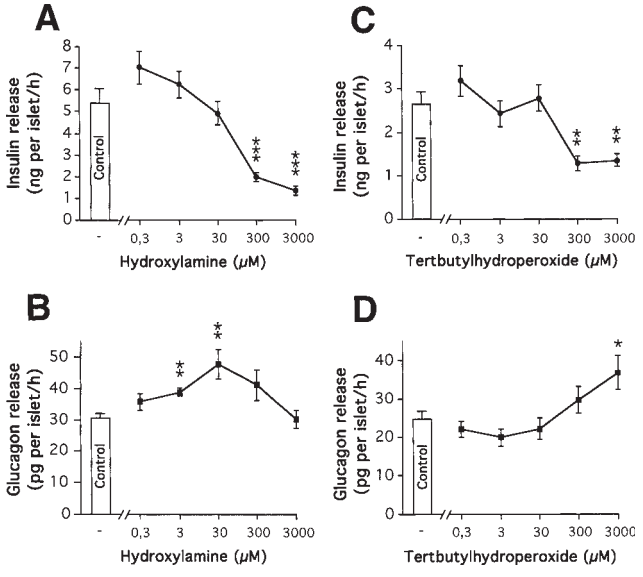

Fig. 1. Effect of different concentrations of the NO donor hydroxylamine **(A,B)** and the "H_2O_2" donor tert-butylhydroperoxide **(C,D)** on insulin and glucagon release stimulated by 16.7 m*M* glucose. Values are means ± SEM from 10-12 incubation vials containing 10 islets each. The incubation period was 60 min. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

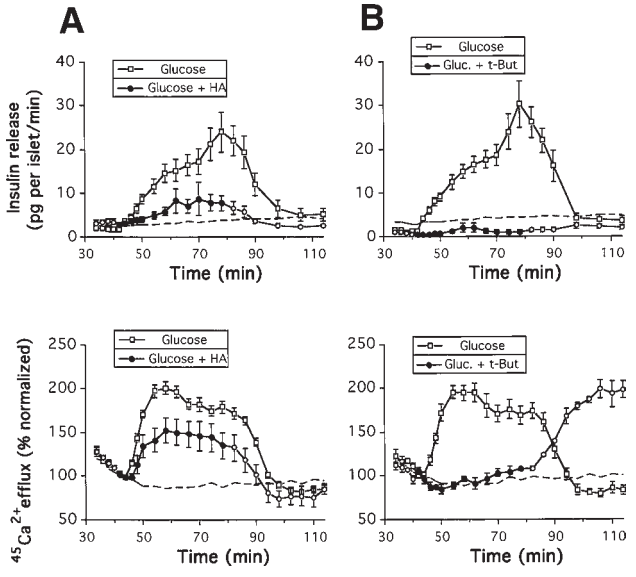

Fig. 2. Effect of 20 m*M* glucose on the dynamics of insulin release **(top panels)** and $^{45}Ca^{2+}$ efflux **(bottom panels)** in the absence or presence of 300 μ*M* hydroxylamine (HA) **(A)** or 300 μ*M* *tert*-butylhydroperoxide (*t*-But) **(B)**. Glucose, hydroxylamine, and *tert*-butylhydroperoxide were introduced at min 40 as indicated. Basal controls (1 m*M* glucose) ($n = 10$) are also shown (– – –). The fractional efflux rate was normalized as described in Materials and Methods. Values are means ± SEM from 3 to 7 perifusion columns in each group obtained from 14 independent experiments.

substrate of catalase, and the intracellular "H_2O_2" donor *tert*-butylhydroperoxide, a substrate of glutathione peroxidase, in the presence of either insulin-stimulating concentrations of glucose or cAMP activating secretagogues.

Results

Influence of NO Donor Hydroxylamine and "H_2O_2" Donor tert-*Butylhydroperoxide on Glucose-Induced Effects on Islet Hormone Release*

Figure 1 illustrates the dose-response effects of different concentrations of hydroxylamine and *tert*-butylhydroperoxide on the release of insulin and glucagon in the presence of high glucose (16.7 m*M*). Glucose-induced insulin release was markedly suppressed by 0.3 and 3.0 m*M* concentrations of both drugs, whereas lower concentrations were without effect (Fig. 1A,C). Glucagon secretion, on the other hand, was significantly enhanced by lower concentrations of hydroxylamine (3 and 30 μ*M*) and a high concentration of *tert*-butylhydroperoxide (3 m*M*) (Fig. 1B,D).

Effects of Hydroxylamine and tert-*Butylhydroperoxide on the Dynamics of Glucose-Induced Insulin Release and $^{45}Ca^{2+}$-Efflux from Perifused Islets*

The next series of experiments was designed to study the time dependence of the effects of the NO donor and the "H_2O_2" donor on glucose-stimulated (20 m*M*) insulin release in a system of perifused islets. These islets were also loaded with $^{45}Ca^{2+}$, which allowed us to follow the $^{45}Ca^{2+}$ efflux pattern. Figure 2 (upper panels) shows that *tert*-butylhydroperoxide (300 μ*M*) (Fig. 2B) induced a very rapid and marked suppression of glucose-stimulated insulin release, whereas hydroxylamine (300 μ*M*) (Fig. 2A) brought about a slower response and a less pronounced inhibition. The pattern of $^{45}Ca^{2+}$ efflux was also differentially affected by the two drugs (Fig. 2; lower panels). Hydroxylamine induced a modest but significant reduction of the glucose-stimulated $^{45}Ca^{2+}$ efflux. *Tert*-butylhydroperoxide, on the other hand, completely suppressed the $^{45}Ca^{2+}$ efflux during the glucose + *tert*-butylhydroperoxide infusion, whereafter the $^{45}Ca^{2+}$ curve started to rise markedly after stopping the infusion.

Effect of Hydroxylamine and tert-*Butylhydroperoxide at 20 mM Glucose on Hormone Release from Islets Depolarized by 30 mM K^+ in the Presence of the K^+_{ATP}-Channel Opener Diazoxide and the NOS-Inhibitor N^G-Nitro-L-Arginine Methyl Ester*

To study whether the effects of the intracellular NO donor hydroxylamine and the intracellular "H_2O_2" donor *tert*-butylhydroperoxide on islet hormone release were exerted independently of membrane depolarization events, we performed a series of experiments in the presence of a depolarizing concentration of K^+ and kept the K^+_{ATP} channels open by diazoxide. In addition endogenous production of NO was prevented by the NOS inhibitor N^G-nitro-L-arginine-methyl ester (L-NAME). Figure 3A shows that the inhibitory effect of 300 μ*M* hydroxylamine on glucose-

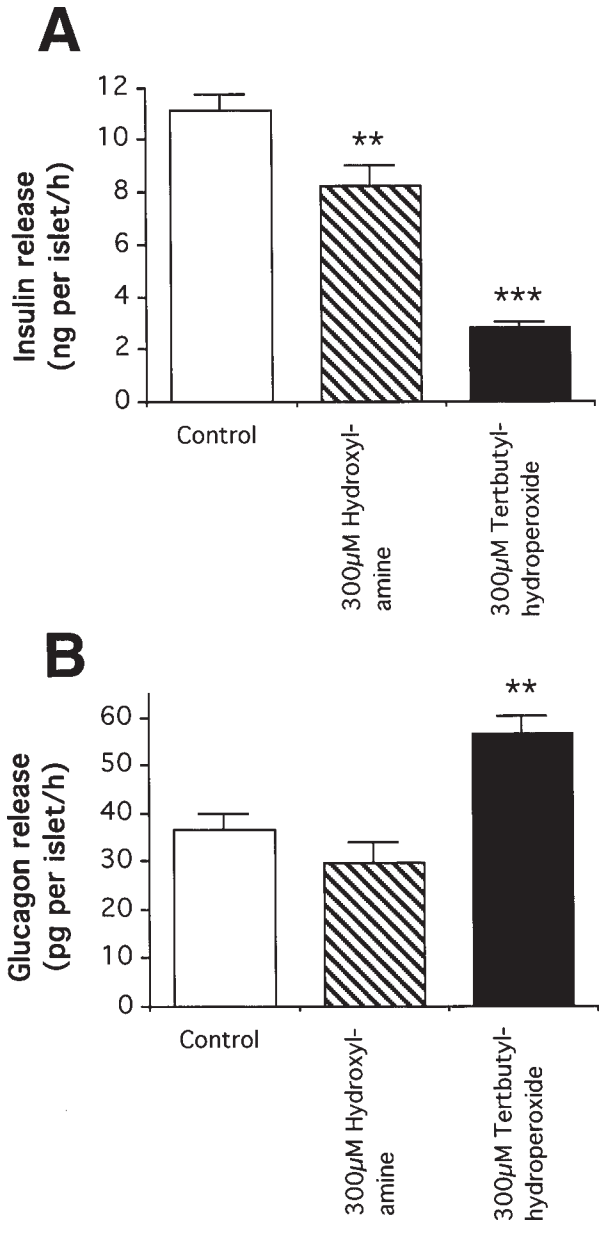

Fig. 3. Effect of 300 μ*M* hydroxylamine (▨) and 300 μ*M* *tert*-butylhydroperoxide (■) on **(A)** insulin and **(B)** glucagon release in the presence of 20 m*M* glucose, 5 m*M* L-NAME, 30 m*M* K^+, and 250 μ*M* diazoxide. Controls (□) were likewise incubated with glucose, L-NAME, K^+, and diazoxide. Values are means ± SEM from 6–7 incubation vials containing 10 islets each. The incubation period was 60 min. $^{**}p < 0.02$; $^{***}p < 0.001$.

stimulated insulin release in depolarized islets was still significant albeit less pronounced than in nondepolarized islets (cf. Fig. 1). In contrast, 300 μ*M* *tert*-butylhydroperoxide showed the same strong inhibitory effect on glucose-stimulated insulin release in depolarized (Fig. 3A) as in nondepolarized islets (Fig. 1C). Similar to the results observed in nondepolarized islets glucagon secretion was not influenced by 300 μ*M* hydroxylamine in depolarized islets (Fig. 3B). However, 300 μ*M* *tert*-butylhydroperoxide induced a significant increase of glucagon release in depolarized islets (Fig. 3B) whereas 3 m*M* of the drug was required in nondepolarized islets (Fig. 1D).

Influence of Hydroxylamine and tert-*Butylhydroperoxide on Insulin and Glucagon Release Stimulated by cAMP-Activating Agents*

To test whether hydroxylamine-derived NO and *tert*-butylhydroperoxide-derived H_2O_2 would affect islet hormone release stimulated by secretagogues activating the cAMP system, we used the adenylate cyclase activator forskolin and the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) at a substimulatory glucose concentration (7 m*M*) *(8)*. Figure 4 illustrates the dose-response effects of hydroxylamine and *tert*-butylhydroperoxide on insulin and glucagon secretion stimulated by forskolin. It is seen from Fig. 4A,C that hydroxylamine potently inhibited forskolin-stimulated insulin release in a dose-related manner, whereas *tert*-butylhydroperoxide displayed an inhibitory action only at the highest concentrations used (0.3 and 3 m*M*). Forskolin-stimulated glucagon release (Fig. 4B,D) was not significantly affected by hydroxylamine at any concentrations used, whereas *tert*-butylhydroperoxide potentiated glucagon release at 30 and 300 μ*M* concentrations. Basal controls in the absence of forskolin are not presented, since we have repeatedly shown that 20 μ*M* forskolin stimulates both insulin and glucagon secretion *(20)*. Neither hydroxylamine nor *tert*-butylhydroperoxide affected the basal secretion of insulin and glucagon at 7 m*M* glucose (data not shown).

A similar series of experiments was then performed with IBMX as a means to raise the cAMP levels and thus activate the cAMP system. Figure 5A,C shows that IBMX-stimulated insulin release was increased by low levels of hydroxylamine (0.3 and 3 μ*M*) but suppressed by 0.3 and 3 m*M* concentrations of the drug. By contrast, *tert*-butylhydroperoxide enhanced insulin release at 0.3–300 μ*M* concentrations and had no effect at 3 m*M*. Glucagon release induced by IBMX (Fig. 5B) was slightly potentiated by low concentrations of hydroxylamine (0.3–3 μ*M*). In contrast, higher concentrations of *tert*-butylhydroperoxide (30–3000 μ*M*) were found to markedly increase IBMX-stimulated glucagon release (Fig. 5D). A very low concentration (0.03 μ*M*) of both free radical donors did not affect IBMX-induced hormone release (data not shown).

Effects of Hydroxylamine and tert-*Butylhydroperoxide on IBMX-Stimulated Hormone Release from Islets Depolarized by 30 mM K^+ in Presence of the K^+_{ATP} Opener Diazoxide and NOS Inhibitor L-NAME*

To compare directly the effects of hydroxylamine and *tert*-butylhydroperoxide on hormone release in K^+-depolarized islets in the presence of 20 m*M* glucose, 30 m*M* K^+, 5 m*M* L-NAME, and 250 μ*M* diazoxide (Fig. 3), we per-

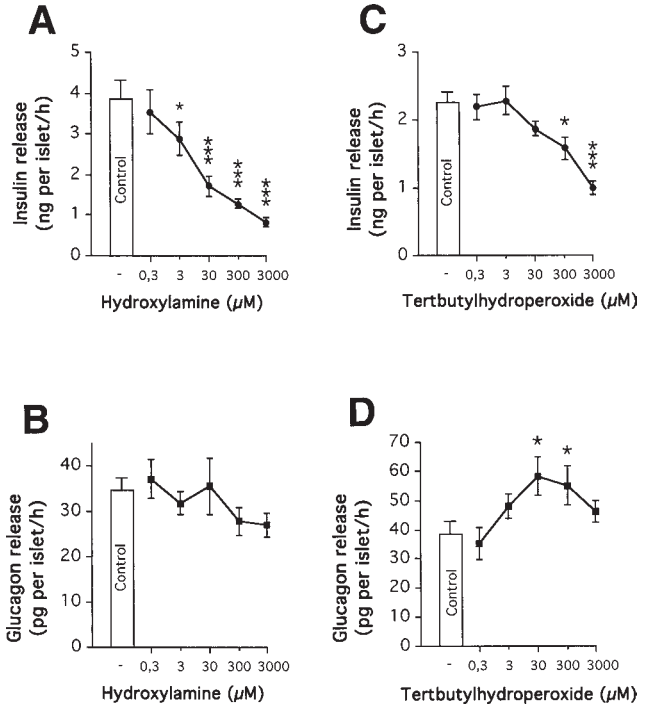

Fig. 4. Effect of different concentrations of the NO donor hydroxylamine **(A,B)** and the "H_2O_2" donor *tert*-butylhydroperoxide **(C,D)** on insulin and glucagon release stimulated by 20 μ*M* forskolin (control) at a substimulatory concentration of glucose (7 m*M*). Values are means ± SEM from 10–12 incubation vials containing 10 islets each. The incubation period was 60 min. **p* < 0.05; ****p* < 0.001.

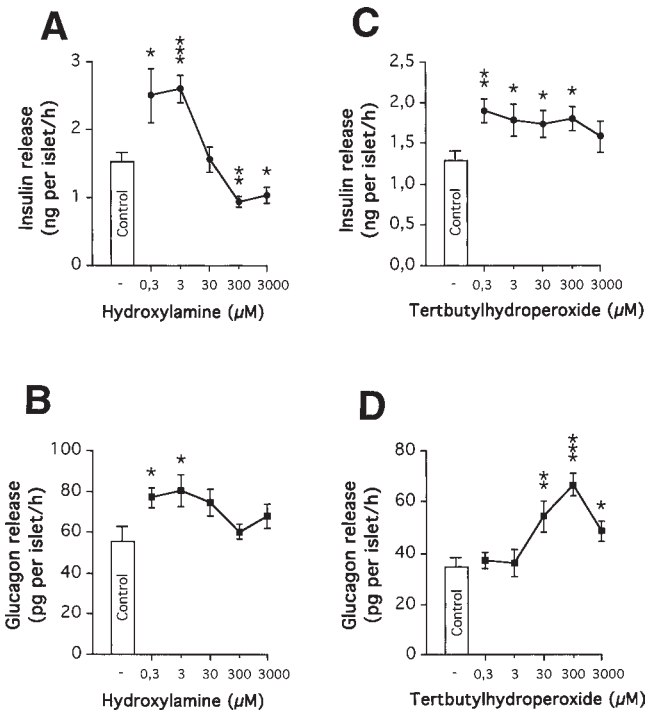

Fig. 5. Effect of different concentrations of the NO donor hydroxylamine **(A,B)** and the "H_2O_2" donor *tert*-butylhydroperoxide **(C,D)** on insulin and glucagon release stimulated by 0.1 m*M* IBMX (control) at a substimulatory concentration of glucose (7 m*M*). Values are means ± SEM from 6–9 incubation vials containing 10 islets each. The incubation period was 60 min. **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

formed a similar experiment in which we replaced 20 m*M* glucose with 0.1 m*M* IBMX and 7 m*M* glucose. Figure 6 shows that the stimulating effects of hydroxylamine (3 μ*M*) on both insulin and glucagon release previously seen in nondepolarized islets (Fig. 5A,B) are still evident in K^+-depolarized islets (Fig. 6), although much less pronounced. By contrast, the stimulating effect of 3 μ*M* *tert*-butylhydroperoxide on insulin release seen in nondepolarized islets (Fig. 5C) did not reach significance in depolarized islets (Fig. 6A). A modest increase in glucagon release was observed in the presence of either of the two free-radical donors (Fig. 6B).

Effect of the NOS Inhibitor L-NAME on IBMX-Stimulated Release of Islet Hormones

Figure 7 shows the effect of the specific NOS inhibitor L-NAME on insulin and glucagon release induced by different concentrations of IBMX at 7 m*M* glucose. Basal controls in the absence of IBMX are also included. L-NAME (5 m*M*) did not significantly affect IBMX-stimulated insulin release at 0.05, 0.5, or 5.0 m*M* concentrations, nor was basal insulin release affected. By contrast, IBMX-stimulated glucagon release (Fig. 7B) was markedly suppressed by L-NAME. No effect by L-NAME on basal glucagon release was found.

In Vivo Effect of L-NAME on IBMX-Stimulated Insulin and Glucagon Response

Figure 8A shows that the insulin secretory response to an iv injection of IBMX was unaffected by a previous injection of L-NAME. By contrast, IBMX-stimulated glucagon response was markedly suppressed by the NOS inhibitor (Fig. 8B). The peak plasma glucagon levels following IBMX injection were 778 ± 101 vs 340 ± 26 ng/L (L-NAME + IBMX) ($p < 0.001$). Basal plasma levels of insulin and glucagon were not significantly affected by the NOS inhibitor (Fig. 8).

Discussion

We have previously shown that both the insulin-producing β-cells and the glucagon-producing α-cells in the endocrine pancreas of the mouse contain a constitutive NO-producing NO synthase (cNOS) *(6,10)* as well as an H_2O_2-producing MAO *(21–23)*. Furthermore, we have also shown that different physiological and pharmacological islet hormone secretagogues were able to modulate the activities of these enzymes in such a way that both insulin and glucagon secretion were influenced *(3,6,8–14,16–19)*. Based on data from early studies *(7)*, we then hypothesized that islet production of low physiological levels of both nitrogen- and

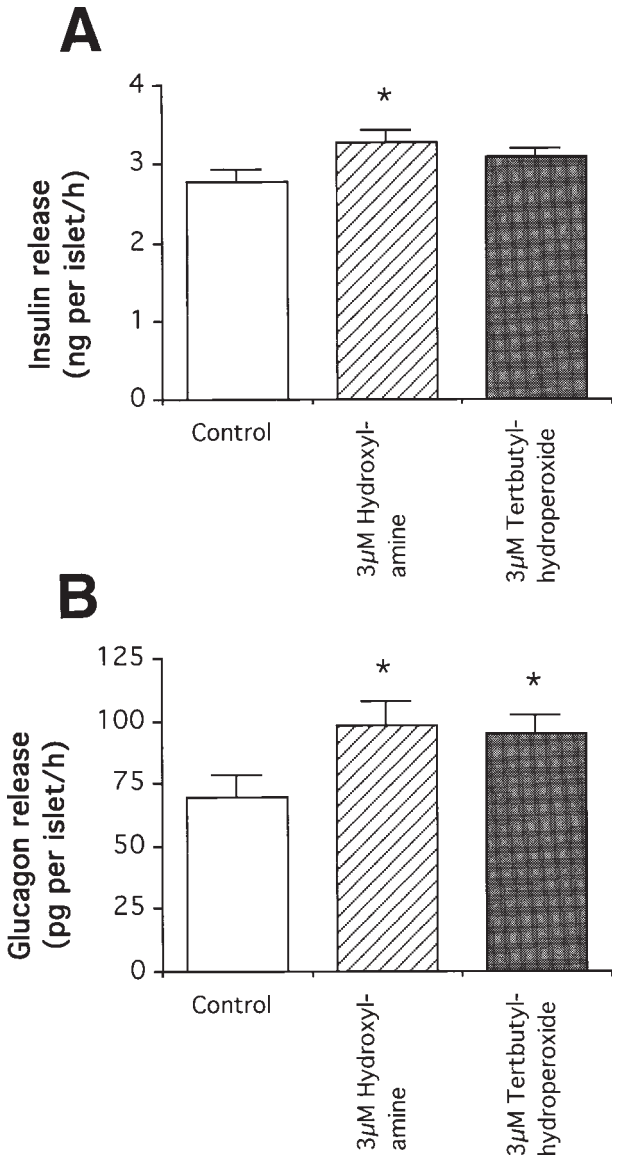

Fig. 6. Effect of 3 μ*M* hydroxylamine (▨) and 3 μ*M* *tert*-butylhydroperoxide (■) on **(A)** insulin and **(B)** glucagon release stimulated by 0.1 m*M* IBMX in the presence of 5 m*M* L-NAME, 30 m*M* K^+, and 250 μ*M* diazoxide at 7 m*M* glucose. Controls (□) were likewise incubated with IBMX, L-NAME, K^+, and diazoxide. Values are means ± SEM from 12 to 13 incubation vials containing 10 islets each. The incubation period was 60 min. *$p < 0.05$.

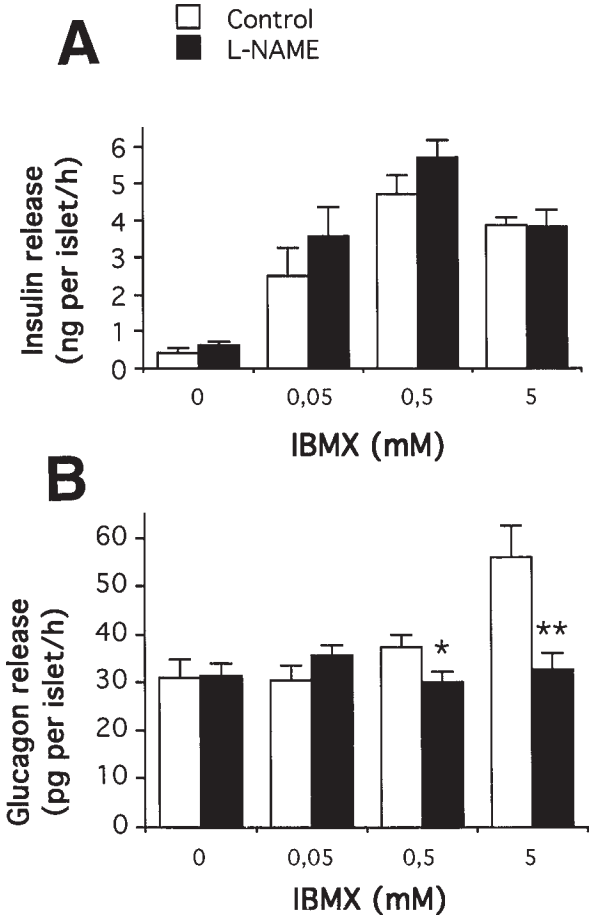

Fig. 7. Effect of different concentrations of IBMX on **(A)** insulin and **(B)** glucagon release in the absence (□) or presence (■) of the NO synthase inhibitor L-NAME (5 m*M*). Glucose concentration was 7 m*M*. Values are means ± SEM from 5 to 7 incubation vials containing 10 islets each. The incubation period was 60 min. *$p < 0.05$; **$p < 0.01$.

oxygen-based free radicals such as NO and H_2O_2 might serve as important modulators of insulin secretory processes. In the present investigation, we approached this issue in an attempt to mimic the physiological intracellular situation by introducing the intracellular NO donor hydroxylamine and the intracellular "H_2O_2" donor *tert*-butylhydroperoxide into isolated islets. The intracellular NO donor hydroxylamine is a known precursor of NO in biological systems, and it has also been suggested to be a physiological intermediate in the conversion of L-arginine to NO *(24)*. Hydroxylamine easily penetrates into cells, where it is oxidized to NO by catalase *(24,25)*. Further, we *(7)* and others *(26)* have previously shown in isolated islets and insulin-secreting cell lines that hydroxylamine greatly elevates the cyclic guanosine monophosphate (cGMP) levels, an effect known as a good marker for NO stimulation in other tissues *(1,2)*. The hydroperoxide precursor *tert*-butylhydroperoxide also penetrates into cells and is metabolized by the glutathione peroxidase–glutathione reductase system *(27)*. We have previously shown that *tert*-butylhydroperoxide did not seem to affect islet cGMP levels, but modestly increased cAMP *(7)*. The present results show distinct differences between the action of hydroxylamine-derived NO and *tert*-butylhydroperoxide-derived "H_2O_2" on the pattern of islet hormone release in the presence of either insulin-stimulating concentrations of glucose or the cAMP activating agents forskolin and IBMX.

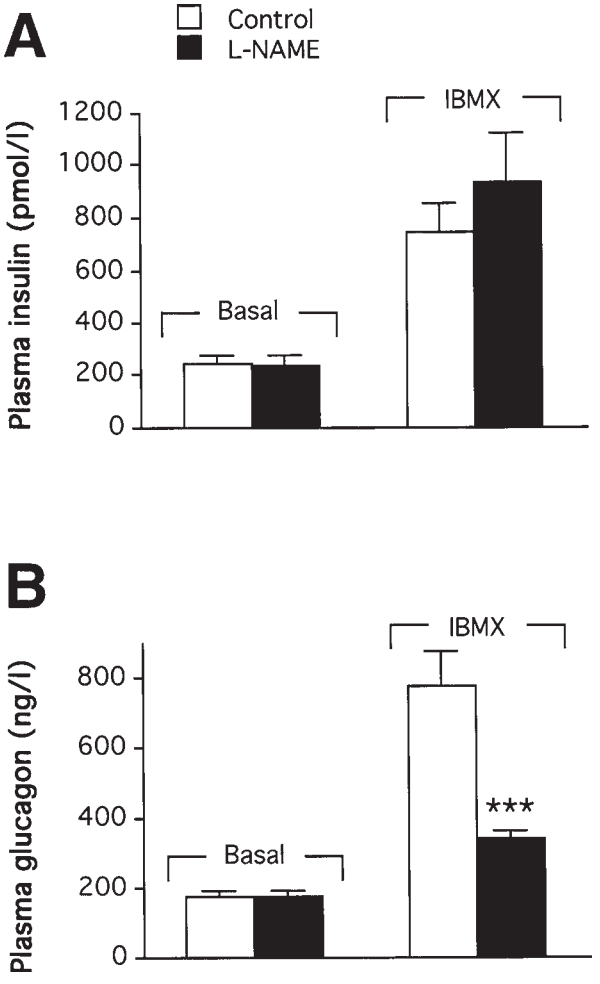

Fig. 8. In vivo effect of L-NAME pretreatment on the **(A)** acute insulin and **(B)** glucagon secretory response to an iv injection of IBMX (45 μmol/kg). L-NAME (1.2 mmol/kg) was administered intravenously 15 s prior to IBMX. Basal controls showing plasma hormone levels after saline + saline and L-NAME + saline are included. Values are means ± SEM from 15 to 16 animals in each group. ***$p < 0.001$. □, Control; ■, L-NAME.

Islet Hormone Release and High Glucose

With regard to the action of the two different free-radical donors on insulin release in the presence of high glucose (Figs. 1–3), our data showed that both hydroxylamine and *tert*-butylhydroperoxide displayed approximately the same inhibitory dose-response characteristics starting their inhibition at a 300 μ*M* concentration. The perifusion studies revealed that the inhibitory action of hydroxylamine on insulin release was strong and sustained and that the inhibitory action of *tert*-butylhydroperoxide was more rapid and also still more pronounced. Furthermore, the $^{45}Ca^{2+}$ efflux data showed distinct differences between the two donors. The marked increase in $^{45}Ca^{2+}$ outflow induced by glucose was only modestly suppressed by hydroxylamine. In contrast, the "H_2O_2" donor totally abolished the $^{45}Ca^{2+}$ efflux during the period of glucose stimulation, whereafter the $^{45}Ca^{2+}$ outflow started to rise continuously and greatly.

The ability of hydroxylamine-derived NO to reduce the glucose-induced outflow of $^{45}Ca^{2+}$ might be the result of a NO-induced opening of the adenosine triphosphate (ATP)-sensitive K^+ channels either indirectly through the suppression of the glycolytic enzyme phosphofructokinase, and thus inhibiting the generation of ATP, or through a direct action of NO on the K^+_{ATP} channels *(28,29)*. Opening of the K^+_{ATP} channels will then result in a decreased entry of extracellular Ca^{2+} and thus a decrease in the glucose-induced insulin release. Further, previous data have shown *(29)* that 1 m*M* hydroxylamine also is able to suppress significantly the increase in β-cell $[Ca^{2+}]_i$ brought about by glucose stimulation. However, because part of the inhibitory effect of hydroxylamine on glucose-induced insulin release was still evident in K^+-depolarized islets in the presence of the K^+_{ATP} channel opener diazoxide, we favor our previous hypothesis *(3,6,7)* that an important action of endogenously derived NO is exerted independently of membrane depolarization events through *S*-nitrosylation of various intracellular targets containing thiol groups critical to the secretory process. The precise nature of these intracellular targets in the stimulus-secretion coupling remains to be elucidated, although an NO-induced derangement of the reduced glutathione/oxidized glutathione (GSH/GSSG) balance, which previously has been suggested to be of utmost importance for glucose-induced insulin secretion *(7,30,31)*, might be one conceivable mechanism. It seems clear, however, that only a part of the effect of hydroxylamine-derived NO might be exerted at the membrane K^+_{ATP} channel. In this context, it should be pointed out that it has previously been shown that the suppressive effect on glucose-stimulated insulin release by a concentration of hydroxylamine as high as 1 m*M* *(29)* was relieved after withdrawal of the drug, thus largely excluding possible toxic effects on the islets in the present study.

Furthermore, the powerful oxidant *tert*-butylhydroperoxide completely abolished both the increased $^{45}Ca^{2+}$-efflux, reflecting glucose stimulation of isotopic exchange between the influx of $^{40}Ca^{2+}$ and efflux of $^{45}Ca^{2+}$, and the insulin release induced by glucose. These data therefore suggest that the *tert*-butylhydroperoxide-derived intracellular evolution of "H_2O_2" inhibited the glucose-induced influx of extracellular Ca^{2+} and thus the release of insulin. Although not strictly comparable, such an explanation would be in accordance with recent data *(32)* showing that exogenously applied H_2O_2 leads to a drop in $[Ca^{2+}]_i$ as a result of the closure of L-type Ca^{2+} channels induced by the hyperpolarization and hence abolishment of glucose-induced insulin release. However, the inhibitory action of *tert*-butylhydroperoxide on glucose-stimulated insulin release was fully operating also in K^+-depolarized diazoxide-treated islets. This observation suggests that *tert*-butylhydroperoxide has a powerful intracellular action as well. It is not inconceivable that the oxidative action of *tert*-

butylhydroperoxide is exerted on "critical" thiol groups situated both in the membrane and intracellularly and that the intracellular action alone is sufficient to suppress totally the glucose-induced insulin release. Such an assumption would be in accordance with a previous observation that *tert*-butylhydroperoxide decreased the GSH/GSSG ratio in rat pancreatic islets *(33)*, especially since GSH is assumed to hold the thiol groups "critical" for glucose-stimulated insulin release in the reduced state *(31)*. The marked increase in $^{45}Ca^{2+}$ efflux seen during the latter part of the infusion time is presumably, at least partly, a reflection of *tert*-butylhydroperoxide-induced mobilization of Ca^{2+} from intracellular stores, which in turn has been shown to lead to an increase in $[Ca^{2+}]_i$ *(32)*. Evidently not even this increase in cytosolic $[Ca^{2+}]_i$ is sufficient to overcome the strong oxidative action of *tert*-butylhydroperoxide. Finally, it should be remarked that previous data have shown that a foregoing perfusion with 2 m*M* *tert*-butylhydroperoxide did not impair a subsequent stimulation of insulin release *(34)*, suggesting no significant injury to the β-cells even at this high concentration of the drug.

There was a marked difference in the effects of hydroxylamine and *tert*-butylhydroperoxide on glucagon secretion in the presence of high glucose. Thus, low concentrations of hydroxylamine (3-30 μ*M*), which did not affect insulin release, did induce a marked increase in glucagon release, whereas higher concentrations were without effect. It should be emphasized that the greatest effect of hydroxylamine-derived NO to stimulate glucagon release is obtained in an incubation medium devoid of glucose *(12)*. Such a stimulatory effect can be suppressed by nutrients known to inhibit glucagon release *(12)* which shows that hydroxylamine actually stimulates glucagon release and is not only preventing the inhibitory effects exerted by these nutrients. Regarding *tert*-butylhydroperoxide, we showed here that it had no effect at low concentrations, but stimulated glucagon release at a 3 m*M* concentration. This stimulating effect was evident also in depolarized islets (at 300 μ*M*), suggesting that *tert*-butylhydroperoxide-induced glucagon release was exerted mainly independently of depolarization events. It seems conceivable that the potentiating effects of NO and hydroperoxide on glucagon release is exerted at the level of secretory granule fusion and exocytosis, since such an action of these free radicals has previously been reported to occur in other cell types with the ability to store and secrete granules and vesicles *(35,36)*. Moreover, the data suggest that the glucagon cell, in this respect, is more sensitive to the action of NO than to hydroperoxide, since the effects of NO were exerted at lower concentrations than those required by hydroperoxide.

Islet Hormone Release and cAMP-Activating Agents

Forskolin-induced insulin release was dose dependently inhibited by hydroxylamine from a concentration of 3 μ*M* and higher, whereas a 100-fold higher concentration was required for *tert*-butylhydroperoxide to be effective. By contrast, low concentrations of both hydroxylamine and *tert*-butylhydroperoxide stimulated insulin release in the presence of the phosphodiesterase inhibitor IBMX. High concentrations of hydroxylamine were inhibitory. Hence, the increase in insulin release induced by raising islet cAMP levels through activation of adenylate cyclase with forskolin, but not through the phosphodiesterase inhibitor IBMX, was quite sensitive to inhibition by hydroxylamine. This finding with regard to forskolin agrees with previous data in other tissues, which showed inactivation of adenylate cyclase by *S*-nitrosylation of essential thiols in the calmodulin-binding domain of the cyclase *(37)*. The observation that IBMX-induced insulin release was increased by low concentrations of both hydroxylamine-derived NO and *tert*-butylhydroperoxide-derived "H_2O_2" is most probably explained by earlier findings showing that NO as well as peroxides have the ability to promote vesicle and granule fusion in other tissues *(35,36)* and therefore might directly and positively modulate the exocytotic process. In this context, note that we have previously shown that stimulation of the cAMP system by the phosphodiesterase inhibitor IBMX, in addition to its phosphodiesterase inhibitory effect, evokes an increased islet MAO activity and thus an increase in H_2O_2 evolution *(6)*. Glucose on the other hand, is known to suppress islet MAO activity and H_2O_2 production *(38)*, but to stimulate NO production *(10,39)*.

The present data are in accordance with previous findings showing that the cAMP system in the β-cell is less sensitive to *S*-nitrosylation or thiol oxidation than transduction signaling induced by thiol-sensitive secretagogues such as glucose, L-arginine, or carbachol *(3,7,10,11,14,16–19,31)*. Interestingly, hydroxylamine-derived NO (but not *tert*-butylhydroperoxide-derived "H_2O_2") slightly but significantly enhanced IBMX-stimulated insulin release in depolarized islets, suggesting that the exocytotic machinery in these IBMX-stimulated islets is highly sensitive to NO.

We have previously shown that inhibition of islet cNOS activity by the selective NOS inhibitor L-NAME potentiates insulin release induced by glucose *(7,10,12,13)*, L-arginine *(3,6,7,9–11,13)*, sulfonylurea *(8)* and cholinergic stimulation *(14,40)* both in vitro and in vivo. Basal nonstimulated insulin secretion at 4 or 7 m*M* glucose, however, was not affected by L-NAME. From the results of the present study, it seems obvious that insulin release induced by IBMX is not significantly affected by the NOS inhibitor L-NAME either in vitro or in vivo. Hence, these results strongly support the data from our experiments with hydroxylamine and again suggest that stimulation of insulin release through the cAMP system is less sensitive to NO-induced *S*-nitrosylation of critical thiol groups than insulin release stimulated by, e.g., glucose, arginine, sulfonylurea, or phospholipase C activation by carbachol *(3,6–14,40)*.

Glucagon release is also stimulated by agents activating the cAMP system. Surprisingly, and in contrast to forskolin-induced insulin release, forskolin-stimulated glucagon release was not affected by hydroxylamine, in spite of NO being a potent inhibitor of adenylate cyclase *(37)*. One explanation might be that the catalase activity, which catalysis the formation of NO from hydroxylamine, is lower in the α- than in the β-cell and then that the NO formed by the action of the peroxisome-located catalase is more efficiently "trapped" intracellularly on its way to the plasma membrane–located adenylate cyclase in the α- than in the β-cell. Another explanation might be that the promotion of granule fusion induced by NO in the exocytotic process strongly counterbalances the adenylate cyclase inactivation. Such an explanation is favored by the finding that both hydroxylamine and especially *tert*-butylhydroperoxide markedly stimulated IBMX-induced glucagon release. An important action of NO in IBMX-stimulated glucagon release is also suggested by our in vivo experiments showing that L-NAME quite efficiently suppressed glucagon release induced by iv injection of the phosphodiesterase inhibitor.

In summary, the data suggest that intracellularly produced NO and hydroperoxide may serve as a potent inhibitory mechanism of the insulin secretory process induced by glucose. The inhibitory effect of these free radicals is presumably exerted by perturbing Ca^{2+}-fluxes and/or through *S*-nitrosylation (NO) and oxidation ("H_2O_2") of thiol groups critical to the secretory process and exerted, at least in part, independently of membrane depolarization events. By contrast, insulin release as well as glucagon release induced by direct cAMP activation through IBMX are increased by both NO and H_2O_2, presumably by enhancing secretory granule fusion and exocytosis, although other mechanisms directly involving cAMP-cGMP pathways cannot be excluded.

Materials and Methods

Animals

Female mice of the NMRI strain (B & K, Sollentuna, Sweden), weighing 25–30 g were used throughout the experiments. They were given a standard pellet diet (B & K) and tap water ad libitum.

Drugs and Chemicals

Collagenase (CLS-4) was purchased from Worthington (Freehold, NJ). Hydroxylamine, *tert*-butylhydroperoxide, forskolin, IBMX, the NOS inhibitor L-NAME and diazoxide were obtained from Sigma (St. Louis, MO). $^{45}Ca^{2+}$ was from Radiochemical Centre (Amersham, UK). Bovine serum albumin (BSA) was from ICN Biomedicals (High Wycombe, UK). All other drugs and chemicals were from British Drug Houses (Poole, UK) or Merck AG (Darmstadt, Germany). The radioimmunoassay (RIA) kits for insulin determinations were obtained from Novo Nordisk (Bagsværd, Denmark), Diagnostika (Falkenberg, Sweden), and Eurodiagnostica (Malmö, Sweden). The antiserum used in the glucagon assay is highly selective against glucagon (Eurodiagnostica).

Experimental Protocol

Preparation of isolated pancreatic islets from the mouse was performed by retrograde injection of a collagenase solution via the bile-pancreatic duct *(41)*. In batch incubation experiments, freshly isolated islets were preincubated for 30 min at 37°C in Krebs-Ringer bicarbonate buffer, pH 7.4, supplemented with 10 m*M* HEPES and 0.1% BSA *(9)*. Each incubation vial was gassed with 95% O_2 and 5% CO_2 to obtain constant pH and oxygenation. After preincubation, the buffer was changed to a medium supplemented with the different test agents, and the islets (10 islets per 1 mL of medium in each incubation vial) were incubated for 60 min in the presence of either 16.7 m*M* or a substimulatory concentration of glucose (7 m*M*) *(8)*. All incubations were performed at +37°C in an incubation box (30 cycles/min). Immediately after incubation, aliquots of the medium were removed and frozen for subsequent assay of insulin and glucagon *(42–44)*.

In the perifusion experiments *(45)*, three groups of islets (100–125) taken from the same pool were transferred to plastic tubes containing 375 μL Krebs-Ringer-buffer supplemented with 20 m*M* glucose and then loaded with 25 μL $^{45}CaCl_2$ (50–100 μCi) that was added from a stock solution with a specific activity of 10–40 mCi/mg Ca^{2+}. The islets were then washed three times with nonradioactive medium and transferred to perifusion columns. The islets were thereby sandwiched between two layers of gel (Biogel P-4, 200–400 mesh; Bio-Rad, Hercules, CA) and perifused at a rate of 0.1 mL/min with the Krebs-Ringer-buffer supplemented with 1 m*M* glucose. Test substances were introduced according to the protocols. The radioactivity lost by the islets was measured in effluent fractions every 2 min (75 μL of the sample was added to 5 mL of scintillation fluid) and counted in a scintillation counter (Packard, Downers Groove, IL). The fractional efflux rate was calculated for each period (radioactivity lost by the islets during the time interval/radioactivity present in the islets during the same time interval) and the mean value was calculated for min 38 and 40 was then normalized to 100% *(10)*. Insulin was determined by RIA *(42)*.

In the in vivo experiments, the various substances were administered intravenously into a tail vein. Control animals were injected with vehicle alone. The volume load was 5–10 μL/g of body weight. Blood samples were taken from the retrobulbar venous plexus using constriction pipets *(46)* at min 6 after injection (peak level of IBMX-induced insulin release). Plasma was separated and then stored at –20°C until determination for immunoreactive insulin and glucagon *(42–44)*.

Statistics

Probability levels of random differences were determined by Student unpaired *t*-test or analysis of variance followed by Tukey-Kramer's multiple comparisons test when applicable.

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