Morphological evidence for the existence of nitric oxide and carbon monoxide pathways in the rat islets of Langerhans: An immunocytochemical and confocal microscopical study

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Abstract

Aims/hypothesis. To map the cellular location of inducible and constitutive nitric oxide synthase and haem oxygenase in rat islets to clarify the morphological background to putative nitric oxide and carbon monoxide pathways.

Methods. Immunocytochemistry and confocal microscopy.

Results. After treatment with endotoxin, immunoreactivity for inducible nitric oxide synthase was expressed in a large number of islet cells, most of which were insulin-immunoreactive beta cells and in single glucagon-immunoreactive and pancreatic polypeptide-immunoreactive cells. Somatostatin-immunoreactive cells lacked immunoreactivity for inducible nitric oxide synthase. In untreated rats, immunoreactivity for constitutive nitric oxide synthase occurred in the majority of insulin-immunoreactive and glucagon-immunoreactive cells, in most pancreatic polypeptide-immunoreactive and somatostatin-immunoreactive cells and in islet nerves. Similarly, immunoreactivity for constitutive haem oxygenase was detected in all four types of islet cells. Endotoxin

treatment did not change the pattern of immunoreactivity for constitutive and inducible haem oxygenase. After treatment with alloxan, insulin-immunoreactivity was observed only in single islet cells, being almost devoid of immunoreactivity for constitutive nitric oxide synthase and haem oxygenase.

Conclusion/interpretation. In vivo endotoxin-induced expression of inducible nitric oxide synthase in insulin-producing and in scattered glucagon-producing and pancreatic polypeptide-producing cells strengthens previous suggestions of a pathophysiological role for inducible nitric oxide synthase in the development of insulin-dependent diabetes mellitus. The presence of constitutive nitric oxide synthase and haem oxygenase in all four types of islet cells, together with recent functional data of ours support roles for nitric oxide and carbon monoxide as intracellular, paracrine or neurocrine modulators of islet hormone secretion. [Diabetologia (1999) 42: 978–986]

Keywords Pancreatic islets, nitric oxide synthase, haem oxygenase, immunocytochemistry, confocal microscopy.

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Abbreviations: CO, Carbon monoxide; HO, haem oxygenase; HO-1, inducible haem oxygenase; HO-2, constitutive haem oxygenase; NO, nitric oxide; NOS, nitric oxide synthase; GLUC,

glucagon; IG, immunoglobulins; INS, insulin; IR, immunoreactive; LPS, lipopolysaccharide (endotoxin); iNOS, inducible nitric oxide synthase; cNOS, constitutive nitric oxide synthase; eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase; PP, pancreatic polypeptide; SOM, somatostatin; ZnPP, zinc protoporphyrin; FITC, fluorescein isothiocyanate conjugated.

Nitric oxide (NO) is a free radical gas that conveys biological information in a way greatly differing from that of the classical transmitters. In the nervous system NO does not act on conventional receptors but through effects on various regulatory processes, intracellularly or in the membrane [1]. The formation of NO is catalysed by the enzyme nitric oxide synthase (NOS), in a reaction in which L-arginine and oxygen are converted to NO and citrulline. There are two major types of NOS enzymes; one inducible isoform *i*NOS, originally described in macrophages and also shown to be expressed in a variety of mammalian tissues among which are the islets of Langerhans [2, 3], and constitutive isoforms (*c*NOS) present in neurons (*n*NOS) and endothelial cells (*e*NOS) [1]. We and others [4–8] have shown previously that the pancreatic islets contain a constitutive NOS as determined by histochemical, immunocytochemical and biochemical methods. Islet *i*NOS has been implicated as an important factor in the pathogenesis of Type I (insulin-dependent) diabetes mellitus [2, 3], whereas islet *c*NOS has been suggested to be involved in the physiological regulation of insulin and glucagon secretion [4–15]. Thus, both *c*NOS and *i*NOS seem to be of great physiological and pathophysiological importance in the pancreatic islets. Constitutive nitric oxide synthase was localised by the use of an antiserum to the neuronal isoform of *c*NOS.

There are diverging results as to the cellular location of the NOS isoforms within the islets, i.e. whether only the insulin producing beta cells or, in addition, other endocrine cell types such as glucagon-producing, somatostatin-producing, and pancreatic polypeptide (PP)-producing cells also contain NOS activity [4–8, 16–18]. Further, in this context, attention has also been drawn to another gaseous molecule, carbon monoxide (CO), since a number of recent studies have shown that CO may serve as a neuronal messenger molecule similar to NO [19–21]. Carbon monoxide is produced by the action of haem oxygenase (HO), at which haem from haemoglobin is degraded to CO and biliverdin [19–22]. The latter compound can then be converted to bilirubin, which is an important antioxidant, the reaction being catalysed by the enzyme biliverdin reductase. Similar to NOS, HO consists of at least two isoenzymes, an inducible (HO-1), and a constitutively expressed isoform (HO-2) [19–21]. Expression of HO-1 is induced by various stress factors, e.g. fever, starvation, oxidative injury. A cytokine-induced expression of a protein (presumably HO-1) has previously been observed in islet tissue, and suggested to be a protective mechanism against oxidative stress [2, 3, 23–25]. Constitutive haem oxygenase is highly expressed in nervous tissue, in which CO may have a transmitter-like function [19, 20] and recent findings of ours suggest a role for CO in the regulation of the release of islet hormones in rats [26].

To further clarify the morphological background to the putative NO and CO functional pathways in the islets of Langerhans, the aim of this study was to map the cellular location of *i*NOS, *c*NOS, HO-1 and HO-2 in islets of normal and alloxan diabetic rats by means of combined immunocytochemical and confocal microscopical methods.

Materials and methods

Tissue handling. Female Sprague-Dawley rats (300–400 g body weight, aged about 3–4 months) were purchased from B&K Universal, Stockholm, Sweden. The animals had free access to water and standard pellets and were used in different experimental groups consisting of four to six animals. One group consisted of rats that received no treatment. One group of rats was given LPS (lipopolysaccharide endotoxin from salmonella typhimurium, Sigma, St Louis, Mo., USA; 10 mg/kg i.p., dissolved in saline) and used after 6 h, at which time there is a high expression of *i*NOS [34]. One group was treated with alloxan (Sigma; 60 mg/kg i.v., dissolved in saline with the addition of a drop of 0.1 N acetic acid to acidify the solution) and killed after 5 days. Plasma glucose was determined by a glucose oxidase method [18, 27] to ensure that the animals had become diabetic. The concentrations of plasma glucose in *freely fed* diabetic animals were 15.1–38.7 mmol/l (total range) compared with 8.9–11.7 mmol/l in normal rats. The rats were anaesthetized with ketamine (100 mg/kg intramuscular; Ketalar, Parke Davis, Barcelona, Spain) and xylazine (15 mg/kg intramuscular; Rompun, Bayer, Leverkusen, Germany) and perfused transcardially through the ascending aorta, first with 100 ml of ice-cold calcium-free Krebs buffer (containing 0.5 g/l sodium nitrite and 10,000 iU/l of heparin), and then with 300 ml of an ice-cold, freshly prepared solution of 4% formaldehyde in phosphate buffered saline (PBS, 0.1 mol/l, pH 7.4). The pancreatic glands were then rapidly dissected out and divided into pieces, which were fixed in the same fixative for 4 hours. After this they were rinsed in ice-cold 15% sucrose in PBS (three rinses during 48 h). The tissue specimens were frozen in isopentane at –40°C and then stored at –70°C. Principles of laboratory animal care (NIH publication No 85–23 1985) were followed and the experimental design was also approved by the animal ethics committee of the University of Lund, Lund, Sweden.

Immunocytochemistry. Cryostat sections were cut at a thickness of 8 µm and thaw-mounted onto chrom alum-coated glass slides and air dried for 30 min to 1 h. To show *i*NOS, *n*NOS, HO-1 and HO-2, sections were pre-incubated in PBS with 0.2% Triton X-100 for about 2 h, and then incubated for 2 days in the presence of rabbit antisera to *i*NOS, *n*NOS, HO-1 or HO-2. The antiserum to *i*NOS (1:500) was generated in rabbits against a 25 amino acid peptide of a cloned inducible NOS from a murine macrophage cell line [28, 29]. The antisera to *n*NOS were generated in rabbits against a 15 amino acid sequence (*n*NOS-15, 1:1280) [30], or a 21 amino acid sequence (*n*NOS-21, peptide 58; 1:2000) [31] from the C-terminal part of a cloned rat cerebellar NOS [32]. The antiserum to HO-1 (1:500; code OSA 100, StressGen Biotechnol, Victoria, Canada) was generated in rabbits against rat liver HO-1. The rabbit HO-2 antiserum (1:1000, code OSA-200; StressGen) was generated in rabbits against rat testes HO-2. After rinsing in PBS (three rinses during 10 min), the sections were incubated for 90 min with fluorescein isothiocyanate conjugated (FITC)

swine anti-rabbit immunoglobulins (IG) (1:80; Dakopatts, Stockholm, Sweden) or Texas red-conjugated affinity purified F(ab')₂ fragments of donkey anti-rabbit IG (1:80; code 711-076-132; Jackson Immuno Research, West Grove, Pa., USA). After rinsing, the sections were mounted in PBS/glycerol with p-phenylenediamine to prevent fluorescence fading [33].

To show two antigens simultaneously [34], sections were incubated overnight with *i*NOS, *n*NOS, or HO-2 antisera (see above), rinsed and then incubated overnight with antisera generated in guinea-pigs to insulin (1:16 000), glucagon (1:4000) and pancreatic polypeptide (1:500) (the latter antisera purchased from Linco Res, St Louis, Mo., USA), and a mouse monoclonal antiserum to somatostatin (1:5 of a prediluted antiserum; cat no 8330-0496, Biogenesis, Poole, England). After rinsing, the sections were incubated for 90 min with FITC goat anti-guinea-pig IG or goat anti-mouse IG (1:80; Sigma, St Louis, Mo., USA), rinsed and then incubated with Texas red conjugated affinity purified F(ab')₂ fragments of donkey anti-rabbit IG (1:80; see above). The sections were rinsed and mounted as described above. An Olympus 3 × 50 fluorescence microscope (LRI Instrument AB, Lund, Sweden) equipped with epi-illumination and appropriate filter settings for Texas Red-immunofluorescence and FITC-immunofluorescence was used for the examinations of the sections [35].

The primary and secondary antisera were diluted in PBS. In control experiments no immunoreactivity could be detected in sections incubated in the absence of the primary antisera or with *n*NOS-15, HO-2, glucagon, somatostatin or pancreatic polypeptide antisera absorbed with excess of the corresponding immunizing antigen (100 µg/ml). No absorption controls could be done with the *i*NOS or *n*NOS-21 antisera as antigenic substances were not available. The characteristics of the *i*NOS and the *n*NOS antisera have been presented previously [29–31]. In control experiments *i*NOS-immunoreactivity was only observed in LPS-induced tissues (macrophages in lung and liver), in which no *n*NOS-immunoreactivity could be seen. As cross reactions to antigens sharing similar amino acid sequences cannot be completely excluded the structures shown are referred to as *i*NOS-, *n*NOS-, HO-1-, HO-2-, insulin-(INS-), glucagon-(GLUC-), pancreatic polypeptide-(PP-), or somatostatin-(SOM-) immunoreactive (IR).

Confocal microscopy. To evaluate whether two immunoreactivities were colocalized within the same cellular structures, sections were analysed in a confocal laser scanning microscope (Multiprobe 2001 TM CLSM; Molecular Dynamics) equipped with an Ar/Kr laser and an inverted Nikon Diaphot TMD microscope as described elsewhere [35].

Results

No *i*NOS-immunoreactivity could be detected in islets of untreated animals. After LPS treatment, *i*NOS-immunoreactivity was expressed in a large number of islet cells, which were diffusely spread over the islets (Fig. 1A, D, G, J). Double immunostaining showed that these cells were also INS-IR, which was further verified by confocal microscopy (Fig. 1C).

Moreover, single *i*NOS-IR cells also displayed GLUC-immunoreactivity and PP-immunoreactivity (Fig. 1F, I) but in most of the GLUC-IR and PP-IR

cells *i*NOS-immunoreactivity was lacking. No *i*NOS-IR cells expressed SOM-immunoreactivity (Fig. 1L). In comparison, treatment with LPS did not seem to change the *n*NOS-immunolabelling pattern with both the *n*NOS antisera used.

Constitutive NOS expressed as *n*NOS-immunoreactivity could be detected in the cytoplasm of most islet cells of untreated animals (Fig. 2A, D, G). The number and distribution patterns of *n*NOS-IR cells were similar with the two NOS antisera used, although the intensity of the NOS-immunofluorescence was weaker with the *n*NOS-21 than with the *n*NOS-15 antiserum. Vessels of capillary size, between the trabecula of islet cells (Fig. 2G) and between exocrine acini, were accompanied by varicose NOS-IR nerve terminals, which were also found around arteries of various sizes. No *n*NOS-immunoreactivity could be detected in endothelial cells of the islet microvasculature.

Double immunolabelling showed that *n*NOS-immunoreactivity was in most INS-IR cells (Fig. 2A–C). In the periphery of the islets there were, however, several *n*NOS-IR cells which lacked INS-immunoreactivity. Most GLUC-IR cells, which were located along the periphery of the islets, were also *n*NOS-IR (Fig. 2D–F), although single GLUC-IR cells were discovered which lacked *n*NOS-immunoreactivity. Pancreatic polypeptide-IR and some SOM-IR cells, which were also located in the periphery of the islets, also expressed *n*NOS-immunoreactivity (data not shown), which in the SOM-IR cells were as far as to the ends of their long and gracile dendritic processes.

In the cytoplasm of almost all islet cells HO-2 immunoreactivity could be detected (Fig. 3 A, D, G, L). Double immunolabelling in combination with confocal microscopy revealed that most INS-IR cells also showed HO-2 immunoreactivity, although there were HO-2 IR cell in the periphery of the islets that lacked INS-immunoreactivity (Fig. 3A–C). Further, along the periphery of the islets there was a broad ring of GLUC-IR, which also were HO-2 IR (Fig. 3 D–F) and dispersed PP-cells and SOM-IR cells with extended processes. These also expressed HO-2 immunoreactivity (Fig. 3G–K, L–N). No HO-1 immunoreactivity could be detected in any type of islet cells.

After treatment with alloxan almost no specific *n*NOS-immunoreactivity and HO-2 immunoreactivity was discovered in most of the damaged beta cells and INS-immunoreactivity was only seen in single islet cells, which were vacuolized and enlarged. In comparison, *n*NOS-immunoreactivity and HO-2 immunoreactivity was well preserved only in non beta cells (GLUC-IR cells and others) (data not shown).

Fig. 1 A–L. Confocal microscopy of rat islets of Langerhans after treatment with LPS. Left panel: red fluorescence in **A**, **D**, **G** and **J** indicating expression of *i*NOS-immunoreactivity (Texas red immunofluorescence). Middle panel: green fluorescence (FITC-immunofluorescence) shows immunoreactivities for insulin (**B**), glucagon (**E**), pancreatic polypeptide (**H**) and soma-

tostatin (**K**). Right panel: overlay picture of **A + B** (= **C**), **D + E** (= **F**), **G + H** (= **I**) and **J + K** (= **L**). Cells showing yellowish fluorescence (arrowheads) indicate colocalization of *i*NOS/insulin (**C**), *i*NOS/glucagon (**F**), and *i*NOS/pancreatic polypeptide (**I**). Bars with numerals indicate lengths (μm)

Fig. 2 A–G. Confocal microscopy of islets of Langerhans of untreated rats. Left panel: red fluorescence in **A** and **D** (Texas Red immunofluorescence) indicates *n*NOS-immunoreactivity. Middle panel: green fluorescence (FITC-immunofluorescence) shows immunoreactivities for insulin (**B**) and glucagon (**E**). Right panel: overlay picture of **A** + **B** (= **C**), and **D** + **E** (= **F**). Yellowish fluorescent cells indicate colocalization of *n*NOS/insulin (**C**) and *n*NOS/glucagon (**F**). Bars with numerals indicate lengths (mm). **G.** Rat islet of Langerhans with *n*NOS-IR varicose nerve terminals (arrowheads) running along vessels of capillary size between endocrine cells. FITC-immunofluorescence. Bar = 100 μ m

Discussion

A characteristic of Type I diabetes is a local inflammatory reaction in the pancreatic islets that are undergoing autoimmune destruction [2, 3]. Nitric oxide has been proposed as a possible mediator in the damage process to the insulin producing beta cells and there is ample *in vitro* evidence that IL-1 and other cytokines are able to induce *i*NOS expression in islet tissue [2, 3]. The pancreatic islet consists, however, of a heterogeneous cell population, making it difficult to localize the cellular source of *i*NOS expression and NO production. It was recently shown that rat islets

exposed to cytokine *in vitro* expressed *i*NOS in their insulin cells whereas the glucagon cells seemed unaffected [7]. No data on the possible existence of *i*NOS in somatostatin cells or PP-cells have so far appeared in the literature.

Lipopolysaccharide (endotoxin) is known to stimulate cytokine production [2, 3]. It is important, however, to note that apart from cytokines, other factors also have been suggested to serve as direct or indirect mediators of effects of LPS. Thus, it is known that LPS may cause the synthesis of reactive oxygen species such as superoxide and hydrogen peroxide, and that NO can combine with superoxide to form the potent oxidizing agent peroxynitrite [2, 3]. Hence, our immunocytochemical data cannot be extrapolated to answer questions on the intimate mechanisms of immune destruction of the islet beta cells. Our results show that after treatment with LPS *in vivo* *i*NOS is expressed in most INS-IR cells and also, although to a much lesser extent, in scattered GLUC-IR and PP-IR cells. No *i*NOS was detected in cells immunostained for somatostatin. Our *in vivo* data of *i*NOS expression in INS-IR cells, agree with findings of previous studies of islets exposed to cytokine *in vitro* [2, 3, 7]. The observation that *i*NOS expression can be elicited in GLUC-IR cells and PP-IR cells has not been described previously. This may be explained by differences between the *in vitro* and the *in vivo* situation and the possible induction by LPS of unknown *i*NOS stimulatory factors. On the other hand, since the fraction of *i*NOS positive cells among the GLUC-IR cell and PP-IR cells is much smaller than among the INS-IR cells, it is not inconceivable that refined techniques such as confocal microscopy are required to observe these *i*NOS positive GLUC-IR cell and PP-IR cells. It is difficult to explain why SOM-IR cells are not influenced by LPS. It has been shown [36, 37] that cells producing somatostatin in contrast to those producing insulin, glucagon and PP are not members of the group of endocrine cells belonging to the amine precursor uptake and decarboxylation series. Whether the ability of endocrine cells to store amine is coupled to that of expressing *i*NOS remains to be explained. If NO finally turns out, however, to be of pathophysiological importance in the development of Type I diabetes, it is notable that not only insulin cells but also GLUC-IR cells and PP-IR cells are able to express *i*NOS. Whether the number of these non-beta cells observed expressing *i*NOS is, however, enough to actually contribute to damaging the beta cells remains to be explained. It is also tempting to speculate that NO derived by the action of *i*NOS in the glucagon cells is at least partly responsible for the increased glucagon secretion in the diabetic condition. Indirectly, such a mechanism might also explain why there is an increased somatostatin response to glucagon in diabetes.

Fig. 3 A–L. Confocal microscopy of islets of Langerhans of untreated rats. Left panel: red fluorescence in **A, D, G** and **J** displays expression of HO-2 immunoreactivity (Texas red immunofluorescence). Middle panel: green fluorescence (FITC-immunofluorescence) shows immunoreactivities for insulin (**B**), glucagon (**E**), pancreatic polypeptide (**H**) and somatostatin

(**K**). Right panel: overlay picture of **A + B** (= **C**), **D + E** (= **F**), **G + H** (= **I**) and **J + K** (= **L**). Cells showing yellowish fluorescence indicate colocalization of HO-2/insulin (**C**), HO-2/glucagon (**F**), HO-2/pancreatic polypeptide (**I**), and HO-2/somatostatin (**L**). Bars with numerals indicate lengths (μm)

Our data show that all four endocrine cell types, i.e. those containing insulin, glucagon, somatostatin and PP showed immunoreactivity for two different antisera of the neuronal isoform of constitutive NOS. As previously discussed it has been known for several years [2, 3] that rat pancreatic islets are able to express the inducible NOS isoform after treatment *in vitro* with different cytokines. The possibility of a constitutive NOS isoform being present has, however, been a matter of debate. We and others [4, 5, 7, 8, 16, 38] have previously observed *c*NOS activity in rat and mouse islet cells by both histochemical (NADPH-diaphorase activity) and immunocytochemical methods. In a recent study, *n*NOS immunoreactivity was shown in rat islets with a weak intensity [38] that was apparently lower than the findings in this study. This is possibly due to differences in methodology and *n*NOS antisera used but also to differences in brightness of the fluorophores of the secondary antibodies [39]. In comparison, other studies carried out with other NOS antisera as well as with the NADPH-diaphorase method were either unable to show *c*NOS activity in rat islet cells or reported that only cells containing somatostatin or islet nerves or both were positive for NOS [40, 41, 42]. The reasons for these discrepancies are not known. The existence of different local isoforms, differences between antisera in recognizing epitopes or differences in methodology are possible explanations. Further, the recent observation that a constitutive *e*NOS is localized to glucagon and somatostatin (but not to insulin cells) in rat islets suggests the possibility that both *e*NOS and *n*NOS are localized in the same cell type [5, 18]. In the present study using two antisera of different sources directed against neuronal NOS, we observed that *n*NOS-immunoreactivity was located in all four types of endocrine cells. Moreover, confocal microscopy disclosed coinciding profiles between cells expressing *n*NOS-immunoreactivity and immunoreactivity for insulin, glucagon, pancreatic polypeptide and somatostatin, respectively. Taken together with the results from previous studies [4–8, 16] this data strongly suggest that a constitutive *n*NOS resides in all these four cell types and in nervous structures as well. Moreover, results of most functional studies on the influence of NOS-inhibitors and NO donors on insulin and glucagon secretion favour NO as an important modulator of the secretory processes of these hormones [4–6, 8–11, 13–15, 43]. Although some early studies [8, 10] and a recent one (using islets from newborn rats) [18] suggested that NO had a positive effect on glucose-stimulated and L-arginine stimulated insulin release, we [4–6, 9, 14, 15, 44–46] and others [13, 43, 47] have repeatedly shown that NO is strongly inhibitory to insulin secretion induced by these secretagogues. The inhibitory effect of NO on insulin release induced by nutrients is probably due to the formation of S-nitrosothiols [48], which impair

important regulatory thiol groups. Thiol groups have long been shown to be essential for stimulus-secretion coupling induced by glucose [49, 50]. In contrast, NO is a positive modulator of glucagon secretion, probably acting by stimulating the cyclic GMP system [5, 6, 44–46].

It has previously been shown that cytotoxicity mediated by cytokine can induce expression of HO-1 in cultured islets [23–25]. That we did not observe any immunocytochemical evidence for HO-1 expression in rats islets after injection with endotoxin does not exclude that HO-1 could be detected by other more sensitive methods but not by immunocytochemistry which might be too insensitive in the present situation. We have very recently obtained evidence for the presence of mechanisms mediated by HO-2 and CO in the release of insulin and glucagon from rat islets [26]. Thus, islet tissue was found to produce large amounts of CO [26]. As this CO production was strongly suppressed by the HO-inhibitor zinc protoporphyrin-IX (ZnPP-IX), and since both insulin and glucagon secretion from intact islets could be suppressed by ZnPP-IX and stimulated by the HO-substrate haemin, we concluded that CO should be recognized as a putative physiologic stimulator of insulin and glucagon release [26]. The data in this study strongly suggest that HO-2 resides in all four types of endocrine cells in rat pancreatic islets and confocal microscopy showed coinciding profiles between cells expressing HO-2 immunoreactivity and immunoreactivities for insulin and glucagon as well as for PP and somatostatin. Hence, the CO-pathway could be of functional significance as an intracellular modulator system, not only for the release of insulin and glucagon [26] but also for the secretion of PP and somatostatin. Thus, from the most recent data and those from this study it seems likely that NO as well as CO formed within the islets of Langerhans are capable of acting both within their cells of origin and also as paracrine, neurocrine or even as endocrine mediators.

In conclusion, we have shown the existence of a morphological substrate for a putative functional role of *i*NOS, *n*NOS and HO-2 as important regulatory enzymes in the physiology and pathophysiology of hormone secretion from the islets of Langerhans.

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