Hypotensive Effect of FAD and Its Mechanism: Involvement of Nitric Oxide Synthase Activation

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Summary Flavin adenine dinucleotide (FAD) is one of the cofactors of nitric oxide synthase (NOS). Recently, we found that FAD acted as an allosteric activator of holo NOS of the neuronal type. In the present study, we obtained the following results in rats: 1) FAD enhanced cyclic GMP (cGMP) production in primary cultures of neuronal cells and its effect was completely abolished by preincubation of the cells with a potent inhibitor of NOS, N^G-monomethyl-L-arginine. 2) FAD injection into the lateral cerebral ventricle decreased the blood pressure and heart rate. This effect was suppressed by preadministration of N^G-monomethyl-L-arginine. 3) The reduction in the blood pressure and heart rate by FAD was accompanied by suppression of the neural activity of the sympathetic efferents to the kidney. This effect was blocked by preadministration of N^G-monomethyl-L-arginine. 4) Intravenous injection of FAD also caused reductions in the blood pressure and heart rate. These results suggest that FAD activates NOS in neuronal cells and that the resultant NO reduces the blood pressure by suppressing sympathetic nerve activity. We also obtained evidence that FAD regulates the cardiovascular system when administered peripherally.

Key Words: cyclic GMP, blood pressure, sympathetic nervous system, nitric oxide, primary cultures of neuronal cells

Nitric oxide (NO) is involved in a variety of physiological events such as neurotransmission [1], vasodilation [2], and the cytotoxic action of the immune system [3] and is produced from L-arginine by nitric oxide synthase (NOS). There

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are reports that its activity is regulated transcriptionally or posttranscriptionally, depending on cell type and isoform, and that its expression in macrophages [4], hepatocytes [5] and vascular smooth muscle cells [6] is induced by cytokines. On the other hand, in neuronal cells and endothelial cells NOS expression was found to be constitutive, its activity depending on Ca^{2+} /calmodulin. In vascular endothelial cells, NOS synthesizes NO in response to various agonists such as acetylcholine [7], ATP, and bradykinin [8] through increases in intracellular Ca^{2+} concentration [9]. In neuronal cells, NOS activity depends on NADPH, tetrahydrobiopterin, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD), as well as on Ca^{2+} /calmodulin. Neuronal NOS contains tightly, but noncovalently, bound FAD [10, 11].

Neuronal NOS is activated when cells are stimulated by glutamate. Glutamate is released from presynaptic terminals and acts on the N-methyl-D-aspartate (NMDA) receptor of the postsynaptic membrane, resulting in increased influx of Ca^{2+} and activation of NOS via the Ca^{2+} /calmodulin system [12, 13]. The NO formed diffuses out from the postsynaptic membrane and activates soluble guanylate cyclase in the presynaptic terminals and astrocytic processes [13], resulting in an increase in intracellular cyclic GMP (cGMP). This suggests that NO might be involved in the regulation of the signal transduction system in the brain. Consistent with this idea, NO was shown to play a critical role in central regulation of sympathetic tone [14, 15].

Recently we found a factor in both rat urine and bovine adrenals that activated neuronal NOS and reduced the blood pressure [16]. We found that this factor resembled FAD in its profile of elution from an anion-exchange column (MonoQ column, Pharmacia, Uppsala), its yellow color, and its loss of activity on treatment with phosphodiesterase. Then we examined the effect of FAD on neuronal NOS. We found that FAD counteracted the inhibition by L-arginine by releasing the negative cooperativity on its activity besides acting as a cofactor (unpublished observation). Here we report the effect of FAD on NOS in primary cultures of neuronal cells *in vitro* and on the blood pressure *in vivo*. We also discuss the relationship between the two phenomena and their possible mechanism.

MATERIALS AND METHODS

Materials. FAD, FMN, riboflavin, calmodulin, poly-L-lysine, urethane, N^G -monomethyl-L-arginine (L-NMMA), and 2',5'-ADP agarose were purchased from Sigma Chemical Co., St. Louis, MO. (6R)-5,6,7,8-tetrahydrobiopterin was purchased from Research Biochemicals International, Natick, MA. Dulbecco's modified Eagle's medium (DMEM) was obtained from Nissui Pharmaceutical Co., Ltd., Tokyo; calf and horse sera were from HyClone Laboratories, Inc., Logan, UT. All other chemicals were from Wako Pure Chemical Industries, Osaka, unless otherwise specified.

Animals. Male Wistar rats, weighing 250–300 g, were used. They were allowed free access to food (laboratory chow pellets, type MF, Oriental Yeast Co., Tokyo) and water, and were kept for at least 1 week before experiments in a room maintained at $24 \pm 1^\circ\text{C}$ and illuminated for 12 h a day (0700–1900 h).

Partial purification of NOS from rat brain. Neuronal NOS was purified as described previously [17], with minor modifications. Briefly, whole rat brains were homogenized in buffer A [25 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 1 mM dithiothreitol and leupeptin (10 mg/ml)] and centrifuged at $30,000 \times g$ for 30 min. The supernatant was subjected to 2',5'-ADP agarose affinity chromatography, and material was eluted with 10 mM NADPH in buffer A. The eluate was applied to an FPLC MonoQ anion-exchange column and eluted with a gradient of 0–0.4 M NaCl in buffer A. By this column chromatography, we obtained a single peak of NOS activity and used this fraction for examination of the effect of FAD.

NOS activity assay. NOS activity was determined by measuring the formation of citrulline. The complete reaction mixture (300 μl) contained a partially purified preparation of NOS (15 μg protein), 50 mM Tris-HCl (pH 7.4), 1 mM NADPH, 1.3 mM L-arginine, calmodulin (6 $\mu\text{g}/\text{ml}$), 1.6 mM CaCl_2 , 50 μM (6R)-5,6,7,8-tetrahydrobiopterin, and flavins. The mixture was incubated for 5 min at 37°C and then boiled for 30 s at 100°C to stop the reaction. The citrulline formed in the reaction mixture was then measured by the method of Schmidt *et al.* [18]. Briefly, 300 μl of the sample was treated with urease (100 mU) for 30 min at 37°C . Then 600 μl of acid solution [$\text{H}_2\text{SO}_4 : \text{H}_3\text{PO}_4 : \text{H}_2\text{O} = 25 : 20 : 55$ (v/v) containing $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (83.4 mg/liter)] and 300 μl of reagent solution [0.01% (w/v) thiosemicarbazide and 0.5% (w/v) diacetylmonoxime] were added, and the mixture was boiled for 15 min and centrifuged for 5 min at $5,000 \times g$. The absorption of the supernatant was then measured at 540 nm.

Neuronal cell culture. Primary cultures of neuronal cells were prepared from the brains of fetal rats at 18 days of gestation as described previously [19]. The cells were suspended at 2×10^6 cells per ml in DMEM containing 5% calf serum and 5% horse serum. Cells in a volume of 2 ml were inoculated into 35-mm tissue culture dishes coated with poly-L-lysine. Two days later, cytosine arabinoside was added at a final concentration of 10 μM . The medium was replaced by DMEM containing 5% calf serum and 5% horse serum without cytosine arabinoside 2 days thereafter. Five hours before cGMP measurement, the medium was replaced by DMEM without sera. When indicated, L-NMMA was added 2 h before the assay at a final concentration of 5 mM. Cyclic GMP production was measured within 7 days after preparation of cells.

Determination of cGMP production. 3-Isobutyl-1-methylxanthine was added to neuronal cells at a final concentration of 0.5 mM 1 min before stimulation. The cells were stimulated with various concentrations of FAD or glutamate in a volume of 100 μl (final concentration, 200 μM) and incubated for 5 min at 37°C . Then they were washed 3 times with ice-cold phosphate-buffered saline (PBS) and collected in 400 μl of PBS. The suspension was mixed with ice-cold

ethanol at a final concentration of 65%, and centrifuged for 5 min at $5,000\times g$. The supernatant was dried under vacuum, and its cGMP concentration was determined by radioimmunoassay with a Yamasa cGMP assay kit (Yamasa Co., Tokyo).

Measurement of intracellular Ca^{2+} concentration. Neuronal cells were cultured on a cover glass with a Flexiperm chamber (Heraeus Co., Germany). The cells were loaded with $5\text{ }\mu\text{M}$ Rhod 2-AM (Dojindo Co., Kumamoto) in buffer B (Krebs-Ringer phosphate buffer containing 5 mM glucose and $500\text{ }\mu\text{M}$ L-arginine). After 30 min, they were washed 3 times with buffer B and incubated in 0.5 ml of buffer B for 30 min. Then the intracellular Ca^{2+} concentration was measured by monitoring the fluorescence intensity by a digital image analyzing technique (ARGUS-200/Ca, Hamamatsu Photonics, Hamamatsu, Japan) as described by Habara and Kanno [20]. Rhod 2 fluorescent images (at 580 nm) were obtained by excitation at 553 nm.

Measurement of blood pressure. Rats were anesthetized with urethane (1 g/kg, i.p.), and a cannula of polyethylene tubing (PE-10, Clay Adams, NJ) was then inserted into the right lateral cerebral ventricle (LCV). Another catheter of polyethylene tubing (PE-50, Clay Adams, NJ) was inserted into the left femoral artery for measurement of the blood pressure. FAD, FMN, or artificial cerebrospinal fluid (aCSF) [21] was introduced into the LCV via the catheter, and the mean arterial blood pressure was recorded using an amplifier (Star Medical, Co., Tokyo) equipped with a transducer to which the other end of the arterial catheter was connected.

For examination of the effect of the intravenous injection on the blood pressure, FAD, FMN, or saline was injected through a catheter inserted into the right femoral vein instead of the LCV.

Measurement of neural activity of sympathetic efferents to the kidney. The neural activity of the sympathetic efferents to the kidney was measured as described previously [22]. Rats weighing about 300 g were anesthetized with urethane, and an LCV cannula was inserted as described above together with a tracheal cannula. Under a dissecting microscope, nerve filaments of the sympathetic efferents to the kidney were isolated from the central cut end to record the neural activity with a pair of silver wire electrodes. Neural activity was amplified, displayed on an oscilloscope, and stored on digital audio tape. The neural activity was analyzed after conversion of the raw data to standard pulses with a window discriminator that separated discharges from background noise. A rate meter with a 5-s reset time was used to observe the time course of change in nerve activity, which was recorded with a pen recorder.

RESULTS

Effects of flavins on NOS

Figure 1 shows the effects of various flavin compounds on NOS activity of a partially purified preparation from rat brain. Consistent with our unpublished

observations, FAD at 10 μM activated NOS about twofold. FMN stimulated NOS activity in a similar manner to FAD, causing greatest stimulation at 10 μM . On the other hand, riboflavin inhibited NOS slightly. FAD, but not FMN, decreased the blood pressure when injected into the LCV and femoral vein, and increased the intracellular cGMP level as described below. Accordingly, only the effect of FAD and its mechanism were examined.

FAD-induced increase in cGMP content in primary cultures of neuronal cells

We examined whether extracellular administration of FAD affected NOS in neuronal cells by using primary cultures of neuronal cells prepared from fetal rat

Fig. 1. Effects of FAD, FMN, and riboflavin on rat brain NOS. A preparation of NOS purified from rat brains by 2',5'-ADP agarose and MonoQ column chromatography was used. Enzyme activities in the presence of various concentrations of FAD (closed circles), FMN (open circles), and riboflavin (open squares) were compared.

Fig. 2. Effect of FAD on cGMP production in primary cultures of neuronal cells. 3-Isobutyl-1-methylxanthine (final concentration, 0.5 mM) was added to primary cultures of neuronal cells 1 min before stimulation. Then cells were stimulated with various concentrations of FAD for 5 min. Values are means $\pm$ SEM of triplicate determinations.

* $p < 0.05$ indicates a significant difference from the control by the t -test.

brain. As NO is known to stimulate cytoplasmic guanylate cyclase [1], the intracellular cGMP level was measured after incubation of the cells with FAD to determine the effect of FAD on NOS activity. As shown in Figs. 2 and 3, cGMP production in primary cultures of neuronal cells was markedly and significantly increased by FAD at 50 μ M, its level being comparable to that observed with glutamate (200 μ M), a physiological agonist for NOS in neuronal cells (Fig. 3). FMN did not affect the intracellular cGMP level (data not shown). Moreover, the finding that FAD-induced cGMP production was completely blocked by the addition of L-NMMA, an inhibitor of NOS, supports the conclusion that cGMP accumulation induced by FAD resulted from activation of neuronal NOS (Fig. 3).

Effect of FAD on the intracellular Ca^{2+} concentration

Based on the proposition that in neuronal cells, activation of NOS is due to elevation of intracellular Ca^{2+} concentration, we examined whether the effect of FAD on cGMP production was mediated by Ca^{2+} . For this purpose we determined the change in Ca^{2+} concentration in neuronal cells after FAD addition by monitoring the fluorescence intensity of a Ca^{2+} indicator, Rhod 2, with a digital image analyzer. We used Rhod 2 because FAD has strong fluorescence at 520 nm (excitation wavelength, 375 nm), which interferes with the signals generated by most Ca^{2+} indicators such as Quin 2, Fra 2 and Fluo 3. Rhod 2 is unique in generating a fluorescence signal at a wavelength of 580 nm, which is not interfered with by the fluorescence of FAD. As a positive control, KCl (final concentration, 75 mM) was added to the cells to depolarize the plasma membrane; and this treatment enhanced the fluorescence intensity of Rhod 2 (Fig. 4B). However, no

Fig. 3. Effects of various agents on cGMP production in primary cultures of neuronal cells. When indicated, L-NMMA (final concentration, 5 mM) was added to primary cultures of neuronal cells 2 h before the experiment. Cells were treated with FAD, glutamate, or DMEM (control) in the presence of 0.5 mM of 3-isobutyl-1-methylxanthine. Final concentrations are shown in parentheses under the names of reagents. Values are means $\pm$ SEM for triplicate determinations. * $p < 0.05$ indicates a significant difference from the control by the *t*-test.

detectable change was observed in the intracellular Ca^{2+} concentration after the addition of FAD (final concentration, $50 \mu\text{M}$) or Krebs-Ringer phosphate (control) (Fig. 4A and C, respectively).

Effects of flavin compounds on blood pressure and heart rate

As both FAD and FMN activated rat brain NOS (Fig. 1), we examined their

Fig. 4. Effect of FAD on the Ca^{2+} concentration in neuronal cells in primary culture. Neuronal cells were cultured on cover glasses with a Flexiperm as described in Materials and Methods. Volumes of $25 \mu\text{l}$ of FAD (final concentration, $50 \mu\text{M}$) (A), KCl (final concentration, 75 mM) (B) and Krebs-Ringer phosphate (control, C) were added to the neuronal cell cultures at the points indicated by arrows. The intensity of the fluorescence of Rhod 2 was monitored by a digital image analyzing technique (Hamamatsu Photonics, Hamamatsu). The figure shows representative results from one of the five experiments performed.

effects on the blood pressure. Injection of FAD into the LCV resulted in a decrease in the blood pressure (Fig. 5A). This hypotensive effect was associated with a reduction in the heart rate (Fig. 5B) and was blocked by preadministration of L-NMMA (Fig. 6A, B). Intravenous injection of FAD also caused the reduction in the blood pressure (Fig. 5D) and heart rate (Fig. 5E). In contrast, neither LCV (Fig. 5C) nor intravenous injection of FMN (Fig. 5F) affected the blood pressure (or heart rate [not shown]). Neither aCSF injection into the LCV nor saline injection into the femoral vein as a control caused any change in the blood pressure and heart rate (data not shown). This experiment was repeated five times with similar results.

Effect on sympathetic efferents to the kidney

The fact that FAD markedly decreased the heart rate suggested that its

Fig. 5. Effects of FAD and FMN on the blood pressure and heart rate. FAD or FMN was dissolved in aCSF at a final concentration of 3 mM and injected in a volume of 10 μ l into the LCV of a rat anesthetized with urethane. Then the blood pressure (A, C) and heart rate (B) were monitored. For peripheral administration, 200 μ l of 100 μ M FAD or FMN dissolved in saline was injected into the femoral vein of a rat anesthetized with urethane. Then the blood pressure (D, F) and heart rate (E) were measured. The result shown is a representative one from five experiments.

hypotensive effect might be due to the decrease in the neural activity of the sympathetic nervous system. Therefore, we next examined whether FAD affected

Fig. 6. Effect of preadministration of L-NMMA on the hypotensive effect by FAD. After $10\ \mu\text{l}$ of L-NMMA ($2\ \mu\text{mol}$) had been injected into the LCV of a rat anesthetized with urethane, FAD ($3\ \text{mM}$, $10\ \mu\text{l}$) was injected. Then the blood pressure (A) and heart rate (B) were monitored. The result shown is a representative one from five experiments.

Fig. 7. Representative tracing depicting the effect of FAD on neural activity of sympathetic efferents to the kidney. First, FAD was dissolved in artificial CSF at a final concentration of $3\ \text{mM}$ and injected in a volume of $10\ \mu\text{l}$ into the LCV of a rat anesthetized with urethane. Then, $10\ \mu\text{l}$ of L-NMMA ($2\ \mu\text{mol}$) was injected into the LCV 20 min before the second FAD injection ($3\ \text{mM}$, $10\ \mu\text{l}$). The electrical activity of the sympathetic efferents to the kidney was monitored.

the neural activity of the sympathetic efferents to the kidney. The sympathetic efferents innervating a unilateral kidney were isolated, and their electrical activity was recorded with a pair of electrodes. As shown in Fig. 7, injection of FAD into the LCV caused a temporary increase followed by a prolonged (more than 1.5 h) suppression of the neural activity of the sympathetic efferents to the kidney. One hundred minutes after the first FAD injection, L-NMMA was administered into the LCV, and an increase in the neural activity of the sympathetic efferents was observed, as reported earlier by Sakuma *et al.* [14]. In such a condition, the second FAD injection caused only a temporary and slight suppression of the neural activity, but the neural activity returned to the elevated level soon. Artificial CSF injection into the LCV as a control did not cause any change in the neural activity of the sympathetic efferents to the kidney except for a temporary increase immediately upon the injection (data not shown). This temporary increase in the neural activity upon injection may be also caused via NO because it was not observed after administration of L-NMMA.

DISCUSSION

The present study indicated that exogenously administered FAD stimulated neuronal cells to induce a variety of responses including hypotension, bradycardia, and a decrease in sympathetic nervous activity.

FAD is a prosthetic group involved in electron-transfer reactions catalyzed by various oxidoreductases. Moreover, we obtained evidence that FAD was not only a prosthetic group, but also an allosteric effector of neuronal NOS (unpublished observation). NOS showed negative cooperativity in the presence of L-arginine at the concentrations more than 15 μM , which is much lower than that in serum (100 μM). FAD activated NOS in a dose-dependent manner by counteracting the negative cooperativity, its effect reaching maximum at 10 μM . This concentration is much higher than that in serum (25 nM) or in brain cells (less than 3.5 μM). In view of these findings, it is possible that FAD functions as an activator of NOS *in vivo*.

The action of NO has been shown to be mediated, at least in part, by cGMP, whose level is regulated by cytoplasmic guanylate cyclase. In the present study, using primary cultures of neuronal cells, we found that FAD stimulated cGMP production and that cGMP production was almost completely blocked by pre-treatment with L-NMMA, a potent inhibitor of NOS. These observations indicate that stimulation of the cells with FAD induced activation of neuronal NOS. Consistent with this conclusion, we recently detected FAD-induced NO production by using an NO-sensitive electrode (Intermedical Co., Tokyo) to measure directly its concentration in the medium of neuronal cell cultures (unpublished observation).

It is unknown how extracellular FAD stimulates NOS. It is thought that FAD is taken up into a cell after it has been hydrolyzed to riboflavin and phosphorylat-

ed to it by an enzyme on the plasma membrane in the small intestine and liver. FAD may cross the plasma membrane by a similar mechanism as in the small intestine and liver, by other transport systems, or by simple diffusion to activate neuronal NOS directly in neuronal cells. The FAD concentration required for cell stimulation was higher than that for direct allosteric activation of NOS (maximal activation, $50\text{ }\mu\text{M}$ for cell stimulation and $10\text{ }\mu\text{M}$ for NOS activation). This difference can be explained by supposing that the efficiency of FAD transport into the cells was low. Another explanation is that FAD interacts with a cell surface receptor molecule to generate intracellular signals leading to stimulation of NOS. Neuronal NOS is a Ca^{2+} /calmodulin-dependent enzyme and is activated by agonists such as glutamate that induce calcium influx into the cells [12, 13]. In the present study, however, FAD did not elicit any detectable change in intracellular Ca^{2+} concentration, judging from the fluorescence of the Ca^{2+} indicator Rhod 2 (Fig. 4). Thus the activation of NOS by FAD seems to be independent of any change in the Ca^{2+} concentration. NOS activity has also been suggested to be regulated by phosphorylation of the serine/threonine residues of the enzyme [11], although the physiological significance of this phosphorylation remains uncertain. NOS has a site for phosphorylation by cAMP-dependent protein kinase. Therefore, FAD may stimulate NOS activity by affecting a protein kinase. Further studies are needed to elucidate details of the activation mechanism of NOS.

In addition to FAD, FMN stimulated neuronal NOS and counteracted the inhibition of the enzyme by L-arginine *in vitro*, as the Hill coefficient was 1.0 in the presence of FMN (unpublished data). However, FMN did not affect the intracellular cGMP level when applied to primary cultures of neuronal cells or the blood pressure when injected into the LCV or femoral vein. Possibly, the efficiency of FMN transport is much lower than that of FAD or FMN has no receptor molecule with which to interact. It should be determined whether the effect of FAD *in vivo* is based on a different mechanism from that operating *in vitro*.

LCV injection of FAD decreased the blood pressure as well as the heart rate, and preadministration of L-NMMA blocked this effect. From these results, it is possible that FAD affected the activity of neuronal cells, especially neuronal cells of the autonomic nervous system via NO. This idea is supported by the findings that LCV administration of FAD reduced the electrical activity of the sympathetic nerves projected to the kidney and that preadministration of L-NMMA blocked this effect. L-NMMA, an NOS inhibitor, was found to increase the activity of the renal sympathetic nerve by inhibiting NOS in the brain [4]. Our findings are consistent with the idea that NO is involved in central regulation of the autonomic nervous system.

In vascular endothelial cells, NO is produced in response to various vasodilators and transmits a signal to smooth muscle cells. We found that intravenous injection of FAD caused a decrease in the blood pressure (Fig. 5). Possibly FAD causes vasodilation through activation of endothelial NOS by a direct action or by an interaction with some cell surface molecules such as adenosine receptors.

The present results indicate that FAD may affect the cardiovascular system, probably by activating NOS in neurons and/or endothelial cells.

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