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# Role of nitric oxide in interleukin 2-induced corticotropin-releasing factor release from incubated hypothalami

(nitric oxide synthase/nitroprusside/$N^G$-monomethyl-L-arginine/carbachol/norepinephrine)

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ABSTRACT **Stimulation of corticotropin-releasing factor (CRF) release from the hypothalamus by interleukin 2 (IL-2) was recently demonstrated. Cytokines induce nitric oxide synthase (NOS), an enzyme that converts L-arginine into L-citrulline and nitric oxide (NO). NO is believed to be responsible for the cytotoxic action of these agents. The constitutive form of NOS occurs in neurons in the central nervous system and NO appears to play a neurotransmitter role in cerebellar and hippocampal function. We explored the probability that IL-2 and synaptic transmitters might release CRF via NO. The effects of L-arginine, the substrate for NOS, and $N^G$-monomethyl-L-arginine (NMMA), a competitive inhibitor of NOS, on IL-2-induced CRF release were studied using mediobasal hypothalami (MBHs) incubated *in vitro* in Krebs–Ringer bicarbonate buffer. L-Arginine did not alter basal and IL-2-induced CRF release after 30 min of incubation but significantly elevated both basal and IL-2-induced CRF release when MBHs were incubated 30 min longer, presumably because the endogenous substrate had been depleted after the initial 30-min incubation period. In 30-min incubations, both carbachol, an acetylcholineomimetic drug, and norepinephrine stimulated CRF release. There was an additive effect of incubation of the MBHs in the presence of carbachol ($10^{-7}$ M) and IL-2 ($10^{-13}$ M). On the other hand, coincubation of MBHs with norepinephrine ($10^{-6}$ M) and IL-2 ($10^{-13}$ M) did not produce any additive effect. Addition of NMMA, an inhibitor of NOS, at 1 or $3 \times 10^{-4}$ M completely suppressed IL-2-induced release of CRF as well as that caused by IL-2 plus carbachol. In contrast, the release of CRF induced by norepinephrine was not blocked by $3 \times 10^{-4}$ M NMMA. The data indicate that IL-2 can activate constitutive NOS leading to increased NO release, which activates CRF release. It appears that NO is also involved in the release of CRF induced by carbachol but not by norepinephrine.**

Recent reports have demonstrated a stimulatory action of interleukin 2 (IL-2) on corticotropin-releasing factor (CRF) release using superfused hypothalami (1) or after static incubation with mediobasal hypothalami (MBHs) (unpublished data). In peripheral tissues, cytokines have been found to induce nitric oxide synthase (NOS), an enzyme that converts L-arginine (L-Arg) into L-citrulline and a reactive gas, nitric oxide (NO) (3, 4). The NO can induce cell death. It takes a number of hours for a cytokine to induce NOS. Consequently, this form of the enzyme has been termed the inducible NOS. A second type of NOS exists in a number of cells throughout the body, for example, in vascular endothelium, and it is termed the constitutive form of the enzyme (3, 4). In contrast to the inducible form, the constitutive form requires activation by an increase in intracellular calcium and its interaction with calmodulin. Both forms of NOS require L-Arg as the substrate (3, 4) and are inhibited by L-Arg analogues, such as $N^G$-monomethyl-L-arginine (NMMA) (3, 4).

The constitutive form of the enzyme has been found in neurons in various loci in the brain and it is abundant in neurons of the supraoptic and paraventricular nuclei (5). The major source of CRF neurons is the paraventricular nucleus although some are also localized in the supraoptic nucleus (6). This similarity in localization raises the possibility that NOS is involved in the activation of CRF release. In the present investigation, we examined the role of NO in release of CRF induced by IL-2 and other neurotransmitters that induce CRF release, such as acetylcholine and norepinephrine (NE). The results indicate that IL-2 induces NO release, which then stimulates CRF release. Carbachol, a cholinergic drug, also appears to induce release of NO, which then stimulates CRF release. However, the stimulatory action of NE on CRF neurons appears to be independent of NO release.

## MATERIALS AND METHODS

Adult male rats (200–225 g) of the Sprague–Dawley (Holtzman) strain (Harlan, Indianapolis) were kept under controlled temperature (24 ± 1°C range) and lighting (hours lights on, 0500–1700) and were given free access to Rat Chow and water.

**Chemicals.** IL-2 (recombinant human IL-2; Collaborative Research) was used. The specific activity was $7.36 \times 10^6$ units/mg of protein and the purity was 95–98%. L-Arg, NMMA, carbachol, and NE were purchased from Sigma. All the solutions were prepared immediately before incubation.

***In Vitro* Incubation.** Animals were killed by decapitation. The MBHs were dissected with vertical cuts along the lateral hypothalamic sulci, the posterior edge of the optic chiasm, and the anterior margin of the mammillary bodies. A horizontal cut 2 mm from the base of the brain was used to separate the island of MBH tissue. Each MBH (14–18 mg) was incubated *in vitro* as reported (7, 8). In brief, one MBH was placed in 250 μl of Krebs–Ringer bicarbonate (KRB) buffer supplemented with 20 μM bacitracin (Sigma) to inhibit peptidases. The tubes were incubated at 37°C in an atmosphere of 95% $O_2$/5% $CO_2$ in a Dubnoff metabolic shaker (50 cycles per min) for a preincubation period of 45 min. Thereafter, the medium was discarded and the tissues were incubated in 250 μl of KRB buffer or KRB buffer containing test substances for 30 min. The medium was then collected and stored at −70°C until assayed for CRF by radioimmunoassay (RIA). In certain experiments, after incubation with test

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Abbreviations: CRF, corticotropin-releasing factor; IL-2, interleukin 2; NO, nitric oxide; NOS, NO synthase; NMMA, $N^G$-monomethyl-L-arginine; MBH, mediobasal hypothalamus; NE, norepinephrine.

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substances for 30 min, a second 30-min incubation with the test substances was performed. Medium at the end of both the first incubation (30 min) and second incubation (30 min) was collected for CRF assay.

**RIA.** CRF concentration in the medium was assayed by RIA, as described (8). A specific rat CRF antiserum (Peninsula Laboratories) was used. CRF was iodinated as described (8). Rat CRF41 (Peninsula Laboratories) was used to construct the standard curve at concentrations of 3.125 pg to 400 pg per tube. Standard and media samples were incubated at 4°C with CRF antibody as described (8). The sensitivity of the assay ($ED_{90}$) was 20 pg per assay tube.

**Statistics.** Statistical probabilities were calculated by analysis of variance with repeated measures followed by the Student–Newman–Keul multiple comparison test. Comparisons resulting in $P < 0.05$ or better were considered significant.

## RESULTS

**Effects of L-Arg on CRF Release.** Because L-Arg is the substrate for NOS, it was important to determine whether it would alter CRF release in the presence or absence of IL-2. IL-2 ($10^{-13}$ M) reliably released CRF in prior experiments. Since L-Arg at concentrations of $10^{-5}$ or $10^{-6}$ M failed to alter basal or IL-2-induced CRF release (data not shown), in the present experiment, the concentration was increased to $3 \times 10^{-5}$ M. As with the lower two doses, $3 \times 10^{-5}$ M L-Arg failed to alter basal or IL-2-induced CRF release after exposure of the MBHs for 30 min (Fig. 1 *Lower*). A slight decrease observed with IL-2 plus Arg was not statistically significant. However, exposure of MBH to L-Arg or IL-2 plus L-Arg for

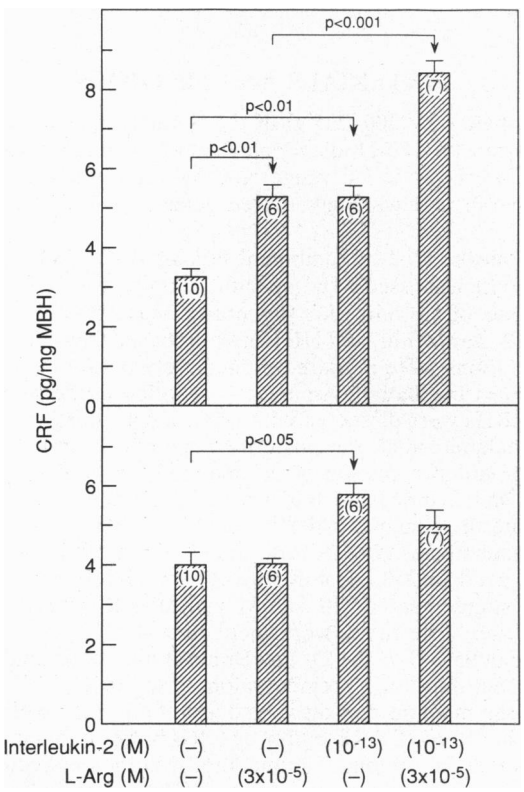


FIG. 1. Effect of IL-2 ($10^{-13}$ M), L-Arg ($3 \times 10^{-5}$ M), or both on the release of CRF from MBHs. (*Lower*) Effect of incubation for 30 min. At the end of this time, the medium was changed and the incubation was continued for an additional 30 min (*Upper*). In this and subsequent figures, the height of the bar represents the mean and the vertical line the standard error of the mean and numbers in parentheses are number of MBH fragments incubated.

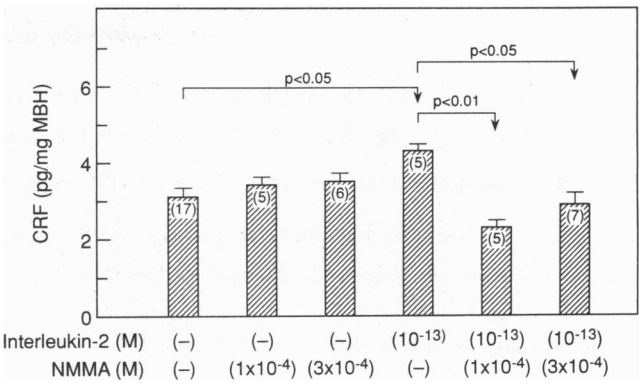


FIG. 2. Effect of NMMA incubated alone or with IL-2 on the release of CRF from MBHs. In this and all subsequent figures, the incubation period was 30 min.

an additional 30 min resulted in a significant increase in CRF release as compared to the control values obtained without Arg (Fig. 1 *Upper*). The increase in CRF release in the presence of both L-Arg and IL-2 was more than additive, which indicates a synergistic effect of L-Arg on IL-2-induced CRF release.

**Effect of NMMA on IL-2-Induced CRF Release.** Addition of NMMA, an inhibitor of NOS, at concentrations of 1 or $3 \times 10^{-4}$ M did not alter basal CRF release (Fig. 2). However, the incubation of MBH with these concentrations of NMMA plus IL-2 completely prevented IL-2-induced CRF release. The decrease observed with $1 \times 10^{-4}$ M was slightly but not significantly greater than that with $3 \times 10^{-4}$ M NMMA.

**Effects of NMMA on IL-2- Plus Carbachol-Induced CRF Release.** Carbachol ($10^{-7}$ M), a muscarinic receptor agonist, evoked a significant increase in CRF release (Fig. 3). Incubation of MBHs with IL-2 plus carbachol markedly increased CRF release. The effect of carbachol plus IL-2 on CRF release was additive. Addition of NMMA (1 or $3 \times 10^{-4}$ M) to MBHs incubated with IL-2 plus carbachol markedly decreased CRF release; however, the decrease caused by $1 \times 10^{-4}$ M NMMA was greater than that observed with $3 \times 10^{-4}$ M.

**Effects of NE or IL-2 Plus NE on CRF Release.** NE ($10^{-6}$ or $10^{-5}$ M) caused a dose-related increase in CRF release (Fig. 4). Coincubation of IL-2 ($10^{-13}$ M) with $10^{-6}$ M NE evoked CRF release comparable to that induced by IL-2 or NE alone.

Incubation of MBH with $3 \times 10^{-4}$ M NMMA plus $10^{-6}$ M NE did not alter CRF release induced by $10^{-6}$ M NE alone (Fig. 5); however, NMMA ($3 \times 10^{-4}$ M) completely blocked the release of CRF induced by IL-2 plus NE.

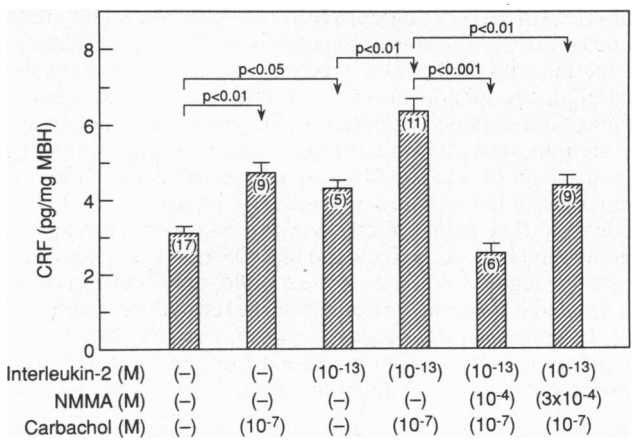


FIG. 3. Effect of NMMA on the response to IL-2 and IL-2 incubated in the presence of carbachol.

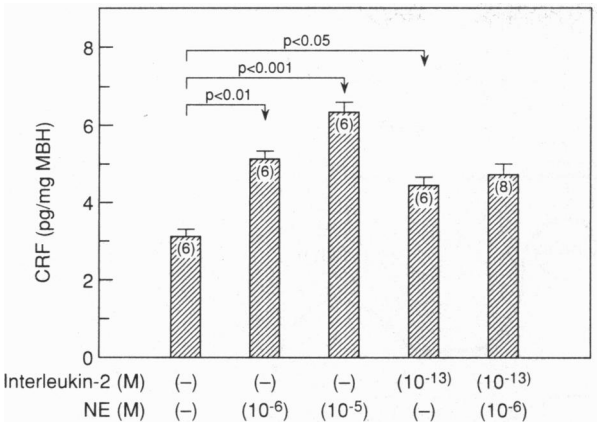


FIG. 4. Effect of two concentrations of NE ($10^{-6}$ and $10^{-5}$ M) incubated alone and the lower concentration of NE ($10^{-6}$ M) incubated with IL-2 ($10^{-13}$ M).

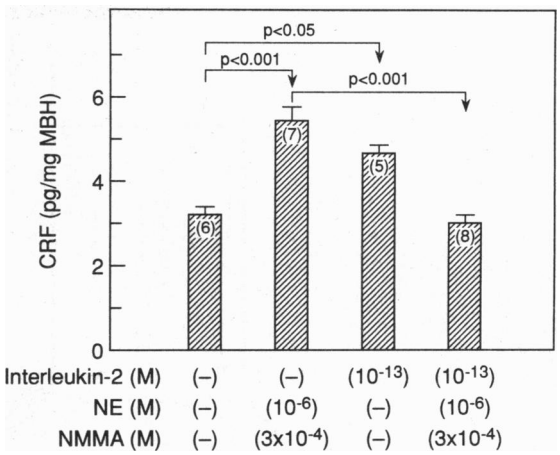


FIG. 5. Effect of NMMA on the response to NE ($10^{-6}$ M) or a combination of IL-2 ($10^{-13}$ M) plus NE ($10^{-6}$ M).

## DISCUSSION

The results clearly demonstrate the stimulatory action of IL-2 on CRF release confirming our previous data (unpublished data) and that of other investigators (1, †). Our data provide evidence that the action of IL-2 to release CRF is mediated via production of NO. Although incubation of MBHs with L-Arg ($3 \times 10^{-5}$ M) for 30 min failed to alter both basal and IL-2-induced CRF release, exposure to L-Arg for an additional 30 min resulted in an augmentation of both basal and IL-2-induced CRF release. The KRB medium provided no amino acids. Apparently, the Arg within the tissue fragment was sufficient to maintain NO formation for 30 min; however, this supply was significantly depleted by the time of the second 30-min incubation and consequently added L-Arg enhanced not only basal but also IL-2-induced CRF release.

Although cytokines have been shown to induce NOS in peripheral tissues, that induction requires 4–18 hr (9), whereas in our experiments effects were obtained within 30 min and, therefore, are presumably caused by activation of the constitutive form of the enzyme. This form of NOS has been found in abundance in the paraventricular nucleus, the major site of perikarya of CRF neurons (5). When the tissue was incubated with NMMA, there was no effect on the resting release of CRF; perhaps, the concentrations of inhibitor we used were too low to block the resting activity of NOS. Alternatively, basal release of CRF during 30 min of incubation is not NOS-dependent. However, the augmentation of CRF release induced by IL-2 was completely abolished. Since NMMA is a competitive inhibitor of NOS (3, 4), this result points to a role for NO in IL-2-induced CRF release. Nitroprusside, which releases NO spontaneously, also increased CRF release, which provides further evidence for the role of NO in CRF release (V. Rettori and S.M.M., personal communication).

IL-2 has several actions in the central nervous system (10, 11) and the presence of specific binding sites in different regions of the brain and its interaction with the cholinergic system has been reported (11). The greatest density of IL-2-like immunoreactivity and IL-2 receptors was localized in the median eminence–arcuate nucleus complex (12). The binding of IL-2 to its receptors has been shown to release γ-interferon (13), which could have activated NOS; however, the rapidity of the effect argues against this possibility. The intracerebroventricular injection of IL-2 dramatically increased, within 10–25 min, the neuron discharge frequency of the paraventricular nuclei (14), where the maximum number of CRF neurons are located (6).

Several investigators have suggested that acetylcholine excites the hypothalamic–pituitary–adrenal axis (15, 16) and muscarinic receptors are known to mediate this stimulation by acetylcholine of CRF release (17). Incubation of MBHs with various concentrations of acetylcholine stimulated CRF release in a dose-related fashion (16–18). This effect was antagonized by the muscarinic receptor antagonist atropine (17). CRF release from the MBH was stimulated by the muscarinic receptor agonist carbachol (17) and this effect was also blocked by atropine (17); the stimulatory action of carbachol observed in our study is in agreement with this report. We found an additive effect of IL-2 plus carbachol on CRF release confirming our earlier data (S.K., K.L., and S.M.M., unpublished data). Addition of 1 or $3 \times 10^{-4}$ M NMMA blocked IL-2- plus carbachol-induced CRF release, which supports the concept that CRF release induced by carbachol as well as IL-2 is brought about by NO. We have also shown that atropine blocks IL-2-induced release of CRF, which points to an essential action of the cytokine on cholinergic neurons, which in turn mediate the release of NO and activation of CRF release from the CRF neurons (S.K., K.L., and S.M.M., unpublished data).

NE was shown to stimulate CRF release (16, 19), although a few reports have suggested inhibition of basal and acetylcholine-stimulated CRF release by NE (18, 20). The reason for the difference in results is not clear; however, the stimulatory action of NE on CRF release observed in this study is in agreement with a previous report (19) that showed that NE induces a dose-related stimulation of CRF release. Our results are also in agreement with another study (21) in which CRF released into the incubation medium was measured by bioassay. The stimulatory effect of NE on CRF release was confirmed by an *in vivo* investigation (22) in which intracerebroventricular administration of NE induced a dose-related elevation of CRF in portal plasma.

In contrast to the results with carbachol, which had an additive effect with IL-2 to increase CRF release, when NE and IL-2 were incubated with MBHs, the release of CRF was similar to that obtained with either IL-2 or NE alone. This suggests either that NE-induced CRF release is blocked by IL-2 or, alternatively, that IL-2-induced CRF release is inhibited by NE. Which of these two possibilities is correct cannot be determined from this experiment. NO does not appear to be involved in the CRF release induced by norepinephrine since $3 \times 10^{-4}$ M NMMA failed to alter NE-induced CRF release. Surprisingly, in view of its failure to alter NE-induced CRF release, NMMA completely sup-

†Calogero, A. E., Luger, T., Gallucci, W. T., Gold, P. W. & Chrousos, G. P., 69th Annual Meeting of The Endocrine Society, June, 1987, Indianapolis, p. 271 (abstr.).

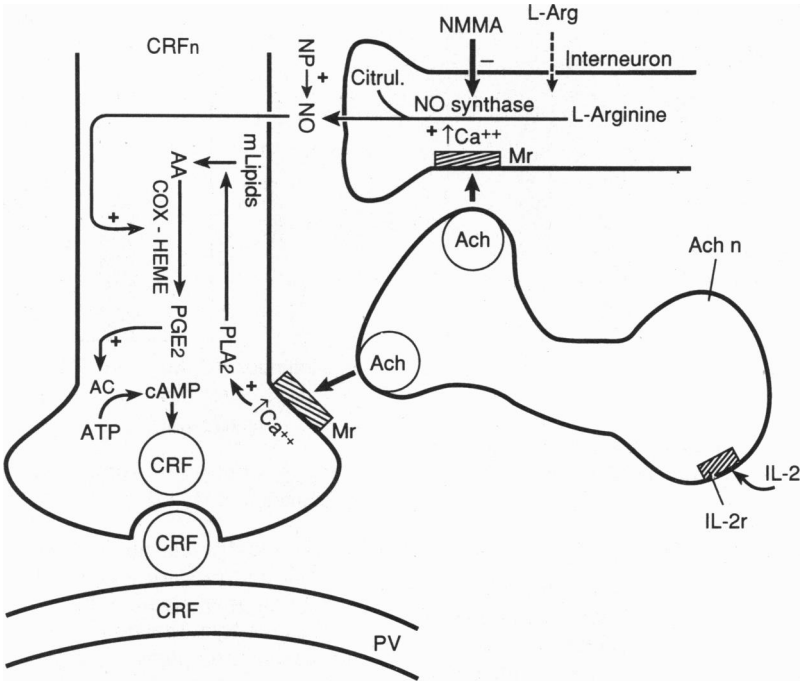


FIG. 6. Schematic diagram of the possible interaction of a cholinergic neuron (Ach n) with an interneuron and CRF neuronal terminals (CRFn) to generate NO, prostaglandin $E_2$ ($PGE_2$), cAMP, and CRF release. For detailed description, see *Discussion*. n, Neuron; mlipids, membrane phospholipids; AA, arachidonic acid; COX-HEME, cyclooxygenase; AC, adenylate cyclase; NP, nitroprusside; citrul, citrulline; $Ca^{2+}$, internal Ca concentration; IL-2r, IL-2 receptor; Mr, muscarinic receptor; $PLA_2$, phospholipase $A_2$; PV, portal vessel; +, stimulation or increase; −, inhibition or decrease.

pressed the release induced by the combination of NE plus IL-2. We believe this result was due to its ability to block the action of IL-2 since the release of CRF in the presence of IL-2 plus NE was the same as that obtained with IL-2 alone. Thus, these data suggest that NO is not involved in NE-induced CRF release and, furthermore, that IL-2 blocks the CRF release induced by NE by a mechanism that does not involve NO.

In nearly all systems studied, the NO pathway involves several cells. We speculate that IL-2 acts on its receptors on cholinergic neurons to cause the release of acetylcholine since IL-2-induced CRF release is blocked by atropine (Fig. 6). The acetylcholine released activates muscarinic receptors on interneurons of unknown type to activate NOS, which causes the release of NO. This NO diffuses to the CRF neuron and activates CRF release by its ability to activate the cyclooxygenase enzyme (23), leading to the generation of prostaglandin $E_2$. Prostaglandin $E_2$ activates CRF via activation of adenylate cyclase and generation of cAMP. cAMP induces exocytosis of CRF secretory granules.

We have reported (23) that NO activates cyclooxygenase *in vitro* in similar hypothalamic fragments. Activation of the constitutive NOS requires an elevation in intracellular calcium that interacts with calmodulin to activate the enzyme. We have previously shown that high potassium concentration in the medium increases CRF release (S.K. and S.M.M., unpublished data), presumably by opening voltage-dependent calcium channels in the interneuron to elevate intracellular calcium, thereby leading to activation of NOS and generation of NO. The NO diffuses into the CRF neuron and activates cyclooxygenase by interaction with the heme group of the enzyme. The cholinergic neuron is also believed to act on the CRF neuron via muscarinic receptors to increase free calcium in the CRF neuron, thereby leading to activation of phospholipase $A_2$. This would cause breakdown of membrane phospholipids to arachidonate to provide substrate for the activated cyclooxygenase enzyme, which results in production of prostaglandin $E_2$. It is possible that there may also be activation of other heme-containing enzymes involved in arachidonic acid metabolism, such as lipoxygenase and epoxygenase, since we imagine that the NO activates cyclooxygenase via the heme group present in these enzymes. Our studies have shown that indomethacin can block the response to IL-2 (S.K. and S.M.M., unpublished data). This is an inhibitor of the cyclooxygenase enzyme and, therefore, this result fits the concept just outlined.

It is likely that other cytokines that stimulate CRF release, such as IL-1, IL-6, γ-interferon, and cachectin, also act via the NO pathway (2).

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