Cocaine potentiates an inflammatory response in C6 astroglia-like cells

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Abstract. Cocaine is a highly addictive drug that mediates its effect through altering dopamine metabolism in the central nervous system (CNS), resulting in a feeling of euphoria. Owing to its high lipophilicity, cocaine easily crosses the blood brain barrier of the CNS and reaches various domains of the brain, where it can trigger cellular damage. Cocaine-induced CNS damage may arise due to increased levels of free radicals and nitric oxide (NO) in immunecompetent astroglial cells. In the present study, the potential ability of cocaine to exacerbate the production of inflammatory products, primarily superoxide free radicals (O_2^-), hydrogen peroxide (H_2O_2) and NO/nitrite (NO_2^-) was examined in rat *C6* astroglia-like cells challenged with lipopolysaccharide (LPS), a bacterial endotoxin, and interferon gamma (IFNγ), a pro-inflammatory cytokine. Furthermore, the role of cocaine in increasing the expression of hypoxia inducible factor-1 (HIF-1α) and vascular endothelial growth factor (VEGF) in cells was also determined. First, the viability of the cells was assessed when treated with cocaine (0.5-7 mM) for 24 and 48 h. The results showed that cocaine toxicity was both time and dose-dependent. In subsequent studies, cells were challenged with or without LPS and IFNγ, followed by co-treatment with cocaine (1-4 mM) for 24 h. Cocaine treatment did not increase O_2^- or H_2O_2 production in the challenged or unchallenged cells. Similarly, cocaine treatment did not increase NO/NO_2^- production in the unchallenged cells; however, NO/NO_2^- levels in the challenged cells was increased 40-50-fold upon cocaine treatment compared with the corresponding unchallenged group. The HIF-1α and VEGF levels were significantly increased in the challenged cells at higher cocaine doses compared with the unchallenged cells. Since high concentrations of NO are associated with inflammation, the high levels of NO production observed in the present study suggested that cocaine may have potentiated the inflammatory response in the challenged *C6* astroglia-like cells.

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Introduction

Cocaine, a potent CNS-stimulant, is abused predominantly in Western countries. Current estimates indicate that >5.9 million Americans used this drug in 2018 (1), and >18 million individuals have used it worldwide (2). Cocaine entry in the brain initiates a variety of responses ranging from toxicity to altered signal transduction in different CNS cell-types (3). However, despite the fact that the brain is composed of neurons, astrocytes, microglia and oligodendrocytes, the majority of *in vitro* or *in vivo* studies with cocaine have been focused on neurons (4,5), partly due to the interactions of cocaine at the signal transduction level.

Astrocytes are one of the most abundant cell-types in the CNS, functioning in neuronal survival and maintenance of fundamental patterns of circuitry (6). It is well established that astrocytes mediate synaptic cross-talk (7), suggesting that astrocytes may be just as important as neurons in research on cocaine abuse. Owing to their abundance in the majority of regions of the brain (8), astrocytes may be the first type of cells to experience the toxic effects of cocaine through decreased mitochondrial membrane potential, resulting in a hypoxia-like state in cells, with regard to being unable to reduce oxygen to water. The hypoxic condition in turn induces the expression of HIF-1α and VEGF in cells and triggers inflammation.

NO is product of inflammatory responses, generated by inducible-nitric oxide synthase (iNOS) in astrocytes (9) under hypoxic conditions (10). *In vivo*, NO is produced from L-arginine by the action of the constitutively active form of NOS due to activation of N-methyl-D-aspartate (NMDA) receptors on glutamatergic neurons. Conversely, iNOS in astrocytes (9) is also involved in NO production from L-arginine through the stimulation of NMDA receptors (11). A previous *in vitro* study showed that cocaine did not stimulate NO production in resting (unstimulated) cells (12). This observation indicated the need of a certain physiological stimulus for NO release.

Astrocytes exposed to external insults/challenges function as immunocompetent cells (13) and release pro or anti-inflammatory signals. Since an unhealthy state of the physical body (for example, bacterial infection) results in altered physiology, it is possible that astrocytes may initiate an immune response by releasing pro or anti-inflammatory molecules (14). Under such a state, it is not clear whether cocaine would potentiate the rate at which NO is produced in the CNS. Excess NO release has been shown to be a contributing factor to several CNS disorders, such as Parkinson's disease (15), schizophrenia or Alzheimer's disease. To date, no studies have determined whether cocaine can potentiate inflammation in astrocytes, to the best of our knowledge.

In the present study, the role of cocaine on the production of different inflammatory products, such as O_2^- free radicals, H_2O_2, NO_2^-, (a stable product of NO), HIF-1α and VEGF was assessed in rat *C6* astroglia-like cells stimulated with LPS (endotoxin) and IFNγ (pro-inflammatory cytokine). These cells are astrocytic in origin and exhibit a high degree of similarity with human astrocytes in terms of gene expression (16), and have been employed frequently in drug abuse studies (12,17-20).

Materials and methods

Materials. DMEM, heat inactivated FBS, Hank's balanced salt solution (HBSS) and PBS were purchased from Bio-Rad Laboratories, Inc. Penicillin/streptomycin sulfate, amphotericin B and L-glutamine were purchased from Mediatech, Inc. Cocaine hydrochloride (Ecgonine methyl ester benzoate; molecular weight, 339.8), trypan blue, sulfanilamide, LPS from *Escherichia coli* serotype 0111:B4, N-(1-naphthyl)-ethylenediamine (NED), phosphoric acid, and EDTA were supplied by Sigma-Aldrich; Merck KGaA. IFNγ protein was obtained from Novus Biologicals Ltd. All other routinely used agents were analytical grade.

Cell culture. *C6* astrocyte-like cell line (CCL-107) was purchased from American Type Culture Collection and maintained as an adherent monolayer culture in complete DMEM in phenol red supplemented with 2 mM L-glutamine, 10% (v/v) FBS, 100 U/ml penicillin, 100 µg/ml streptomycin sulfate and 0.25 µg/ml amphotericin B. Cells were grown in a humidified incubator at 37˚C with 5% CO_2, and sub-cultured twice a week. For cytotoxic studies, the culture was harvested by treating with 0.05% EDTA in PBS for ≤2 min, resulting in a single cell suspension. Cell count was assessed using 0.4% trypan blue dye exclusion assay (27˚C for 1 min) using a hemocytometer under a light microscope (magnification, x20).

Viability assay. Cells ($5x10^4$/well) in 96-well plates were treated with cocaine (1-4 mM) for 24 h. Cocaine stock, working stocks and treatments were prepared as described previously (17). Cell viability was evaluated using a crystal violet dye uptake assay as described previously (17). At the end of treatment, 100 µl 0.25% glutaraldehyde was added per well and incubated for 30 min. After washing and air drying of plates, the dye was extracted with 100 µl 50 mM sodium phosphate mono basic solution, containing 50% ethyl alcohol. The plates were gently vortexed and the optical density (OD) measurements of incorporated dye in viable cells were measured at 540 nm using a microplate spectrophotometer (Bio-Tek Instruments Inc.).

Superoxide assay. Cells ($5x10^4$/well) in 96-well plates in phenol red free media were treated with LPS (0.2 µg/ml), IFNγ (6 µg/ml) and cocaine (1-4 mM) for 24 h. Production of superoxide radicals from cells was detected using a cytochrome *c* reduction assay as described previously (21). Briefly, at the end of the 24 h treatment, the supernatant from samples was added to an equivalent volume of cytochrome *c* from horse heart (Sigma-Aldrich; Merck KGaA) prepared in PBS (final working concentration: 160 µM). Then the samples were incubated for 35 min at 37˚C with 5% CO_2. OD measurements of samples were obtained at 550 nm using a UV microplate spectrophotometer.

Hydrogen peroxide assay. Cells ($5x10^4$/well) in 96-well plates in phenol red free media were treated as described above, and H_2O_2 production was assessed using a previously described method with some modifications (22). After 24 h of treatment, sample supernatants were assessed for H_2O_2 production using a peroxidase linked continuous assay. The chromogenic solution contained (final working concentration) 1 mM vanillic acid, 500 µM 4-aminoantipyrine and horseradish peroxidase (4 U/ml) in PBS with 2 mM HEPES (pH 7.4). The chromogenic solution was added to each sample and incubated for 10 min at 37˚C. Samples were analyzed at 490 nm in a UV micro plate spectrophotometer (Bio-Tek Instruments Inc.). Controls and blanks were measured simultaneously, and subtracted from the final value to eliminate interference.

NO/NO_2^- determination. In order to determine the optimum concentrations of IFNγ (2.6, 3.3, 4.0, 4.6, 5.3, 6.0, 6.6 7.3, 8.0 and 8.6 µg/ml) or LPS (2.6, 3.3, 4.0, 4.6, 5.3, 6.0, 6.6 7.3, 8.0 and 8.6 µg/ml), a dose-response study was initially performed in 96-well plates. Then, the role of cocaine on NO generation was studied by treating the cells in phenol red free media as described above. At the end of treatments, Griess reagent (mixture of an equal volume of 1% sulfanilamide in 0.5 N HCl and 0.1% NED in deionized water) was added directly to the cells under reduced lighting at room temperature. The plates were read at 540 nm on a UV microplate spectrophotometer. Controls and blanks were measured simultaneously, and subtracted from the final value to eliminate interference. A standard curve was generated from a range of dilutions of sodium nitrite (1-100 µM) prepared in the plating medium.

HIF-1α ELISA. For quantification of HIF-1α release in cell lysates, the cells ($5x10^4$/well) were seeded in 96-well plates with DMEM. Supernatants from resting (unstimulated) and stimulated (with LPS and IFNγ) cells after 24 h cocaine exposure at different concentrations (1-4 mM) were discarded, and ice cold PBS was immediately added to each well, decanted and 100 µl cell lysis buffer (Abcam) supplemented with 1:500 µl protease inhibitor (cat. no. ab65621; Abcam) and N-methoxyoxoacetyl-glycine methyl ester (Sigma-Aldrich; Merck KGaA; cat. no. D3695, lot#:064M4728V) was added to each well. After three freeze/thaw cycles at -80˚C, the content of each well was centrifuged at 100 x g for 5 min at 4˚C. Then,

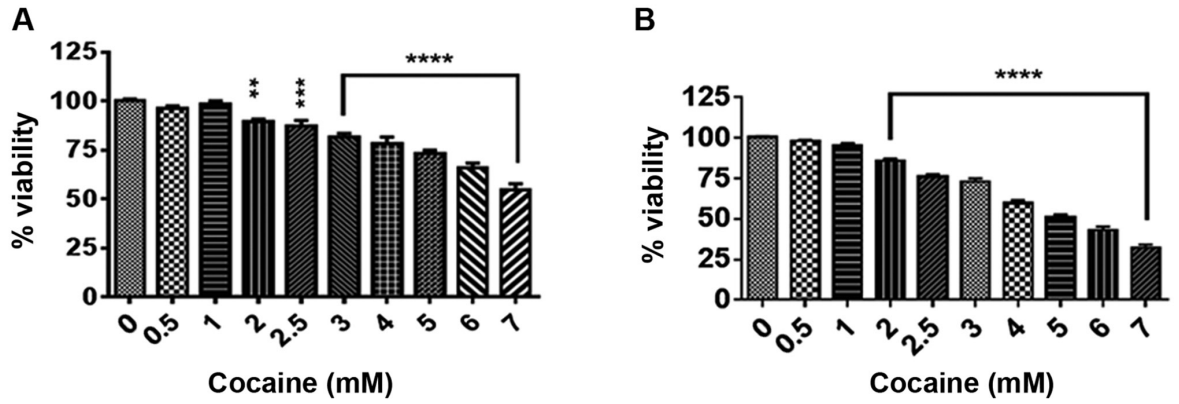

Figure 1. Effect of cocaine on the viability of cells. Cells were treated with various concentrations of cocaine for (A) 24 or (B) 48 h in 96-well culture plates. n=8. **P<0.01, ***P<0.001, ****P<0.0001 vs. respective control (0 mM).

90 μl supernatant (centrifuged vial) was added to each ELISA well. ELISA (kit sensitivity, 0.78 pg/ml) was performed using a HIF-1α ELISA kit (CUSABIO; cat. no. E08540r) according to the manufacturer's protocol. Briefly, 90 μl supernatant from samples and standards were added to 96-well plates pre-coated with the capture antibody. After incubation for 2 h at 37˚C, the supernatant from each well was decanted and 100 μl of the prepared biotinylated primary antibody mixture was added. After 1 h of incubation at 37˚C, all wells were washed 3 times with 300 μl 1X wash buffer provided in the kit. The wash buffer was discarded and 100 μl prepared secondary antibody, HRP-avidin, was added to each well and incubated for 1 h at 37˚C. Subsequently, all wells were washed 5 times with 300 μl 1X wash buffer provided in the kit. After removal of the washing buffer, 90 μl of the color developing agent was added to each well and incubated at 37˚C for 25 min. Finally, 50 μl stop solution was added to each well and the absorbance was read at 450 nm using a UV microplate reader.

VEGF ELISA. For quantification of VEGF release in cell lysates, the cells ($5x10^4$/well) were seeded in 96-well plates with DMEM. After discarding the supernatant, the cell lysate was prepared as described for the HIF-1α ELISA. ELISA (kit sensitivity, 20 pg/ml) was performed using a VEGF ELISA kit (Boster Biological Technology; cat. no. EK0540) according to the manufacturer's protocol. Briefly, 100 μl of the samples and standards were added to 96-well plates pre-coated with the capture antibody. After incubation for 90 min at 37˚C, the supernatant from each well was decanted and 100 μl of the prepared biotinylated primary antibody mixture was added. After 1 h of incubation at 37˚C, all wells were washed 3 times with 300 μl 0.01 M PBS. The wash buffer was discarded and 100 μl of the prepared secondary antibody, Avidin-Biotin-Peroxidase Complex, was added to each well and incubated for 30 min at 37˚C. Subsequently, all wells were washed 5 times with 300 μl 0.01 M PBS. After removal of the washing buffer, 90 μl color developing agent was added to each well and incubated at 37˚C for 25 min. Finally, 100 μl stop solution was added to each well and the plate was read at 450 nm using an UV microplate reader.

Statistical analysis. Data are presented as the mean ± standard error of the mean. Differences between groups were compared using a one-way ANOVA followed by a post-hoc Dunnett's tests. Data analysis was performed using GraphPad Version 6 (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Cocaine decreases cell viability. Initially the effect of treatment with cocaine at concentrations ranging from 0.5 to 7 mM for 24 and 48 h on cell viability was assessed. The selection of cocaine concentrations was based on earlier reports (20,23), and the lag period and doubling time of these cells (17) were used as the criteria for treatment durations of 24 and 48 h. Compared with the corresponding controls, cocaine significantly decreased the cell viability at these time points (Fig. 1A and B; P<0.01 or P<0.001). The LC_{50}, where 50% of cells were killed by cocaine, was determined to be between 4.5 and 3.8 mM at 24 and 48 h, respectively.

O_2^- free radical and H_2O_2 production. Cells treated with cocaine for 48 h exhibited a higher rate of death compared with 24 h of treatment (Fig. 1A and B); in order to prevent severe loss of cells, 24 h of treatment was selected as the end point for subsequent experiments. The effect of cocaine on the production of O_2^- free radicals and H_2O_2 in cells with or without LPS and IFNγ stimulation was determined. It was found that there was no difference between the unstimulated or stimulated groups irrespective of cocaine treatment (P>0.05) for either of the products (Fig. 2A and B).

NO/NO_2^- production. Next NO generation in cells was observed following stimulation and treatment. To determine the optimum concentrations of LPS and IFNγ, the cells were initially treated for 24 h with increasing concentrations of IFNγ against a fixed concentration (2.5 μg/ml) of LPS (Fig. 3A). Similarly, a dose-response experiment was performed for LPS was performed for 24 h against a fixed concentration (2.5 μg/ml) of IFNγ (Fig. 3B). It was found that 0.2 μg/ml LPS and 6 μg/ml IFNγ were ideal to stimulate NO/NO_2^- production without compromising the cell viability (data not shown), consistent with a previous study (24). Based on this, the cells were stimulated with LPS and IFNγ at these doses, and treated with cocaine for 24 h. Lack of LPS and

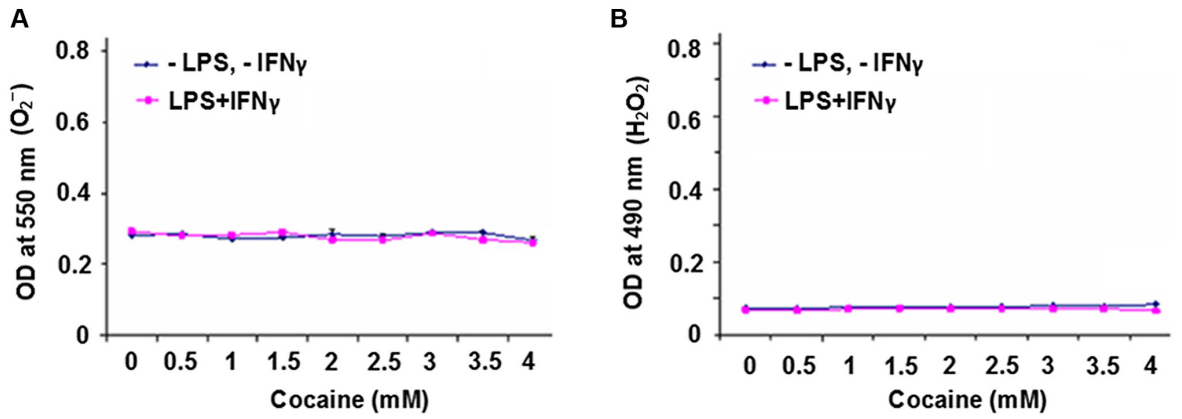

Figure 2. Effect of cocaine on the formation of inflammatory products. (A) O_2^- free radicals or (B) H_2O_2 were measured in cells cultured in the media lacking phenol red and treated with various concentrations of cocaine in the absence or presence of both LPS (0.2 μg/ml) and IFNγ (6 μg/ml) for 24 h. n=4, not significant compared to control (0 mM). LPS, lipopolysaccharide; IFNγ, interferon-γ.

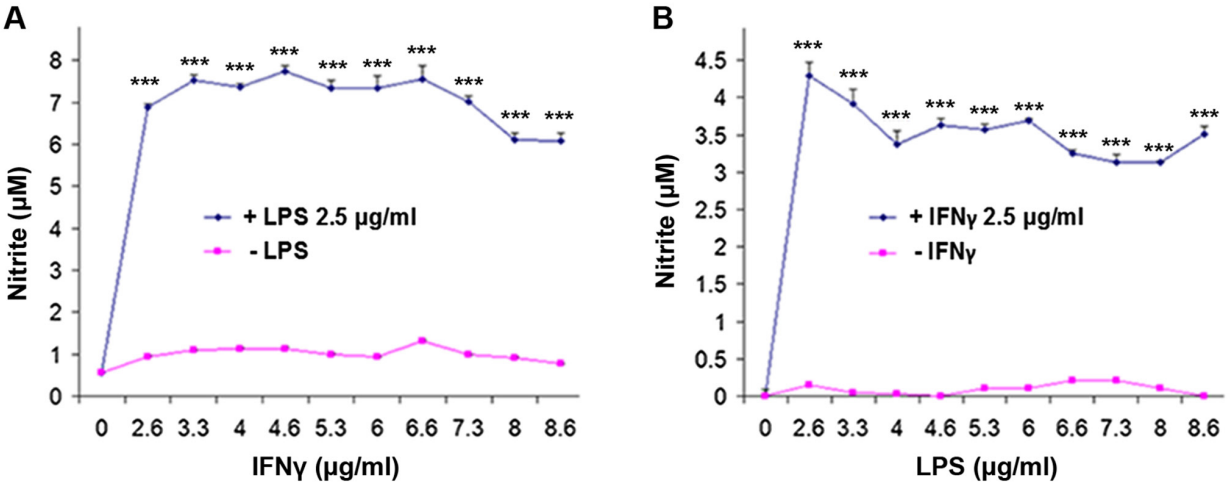

Figure 3. Standardization of LPS and IFNγ. Cells cultured in the media lacking phenol red were treated with (A) various concentrations of IFNγ and 2.5 μg/ml LPS, or (B) various concentrations of LPS and 2.5 μg/ml IFNγ for 24 h. n=4. ***P<0.001 vs. respective control (0 μg/ml). LPS, lipopolysaccharide; IFNγ, interferon-γ.

IFNγ stimulation did not result in altered NO/NO_2^- production in the cocaine treated cells (Fig. 4); however, when stimulated with LPS alone, cocaine at different concentrations increased NO/NO_2^- production significantly in a dose-dependent manner compared with the unstimulated cocaine-treated cells (P<0.01), corroborating a previous report on LPS-induced NO/NO_2^- production (25). Conversely, IFNγ-stimulated cells did not produce NO/NO_2^- following cocaine treatment. However, LPS and IFNγ-stimulated cells treated with cocaine showed a significant increase in NO/NO_2^- production (40-50x; absorbance, 0.04 to 0.05, respectively; Fig. 4) compared with the unstimulated cells (absorbance, 0.001; Fig. 4; P<0.001).

HIF-1α levels are increased by treatment with cocaine in the stimulated cells. In the absence of external challenge to the cells, there was no significant increase in HIF-1α with cocaine treatment at 3 and 4 mM (Fig. 5). However, when the cells were challenged with LPS and IFNγ, the HIF-1α levels in cells treated with 4 mM cocaine increased significantly by 5-fold (P<0.05). The average increase was 25.3±4.9 pg/ml of the control (5.1±2.1 pg/ml).

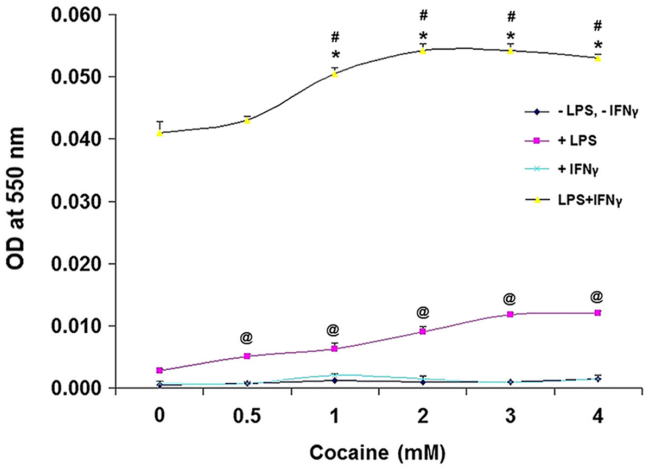

Figure 4. NO/NO_2^- production in cells. Cells in the media lacking phenol red were treated with various concentrations of cocaine in the absence or presence of LPS (0.2 μg/ml) alone or IFNγ (6 μg/ml) alone, or both together for 24 h in 96-well culture plates. n=4. @P<0.01 vs. respective 0 mM cocaine with LPS alone. *P<0.01 vs. respective 0 mM cocaine with LPS and IFNγ. #P<0.001 vs. stimulated cocaine treated cells with un-stimulated cocaine treated cells. LPS, lipopolysaccharide; IFNγ, interferon-γ.

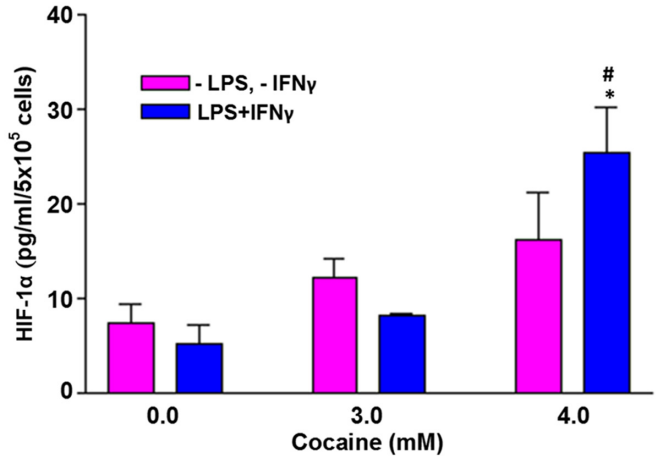

Figure 5. Detection of HIF-1α using ELISA. Cells in the media lacking phenol red were treated with various concentrations of cocaine in the absence or presence of LPS (0.2 μg/ml) and IFNγ (6 μg/ml) together for 24 h in 96-well culture plates. n=3. *P<0.01 vs. respective 0 mM cocaine with LPS and IFNγ. #P<0.001 stimulated cells treated with 4 mM cocaine vs. un-stimulated cells treated with 4 mM cocaine. LPS, lipopolysaccharide; IFNγ, interferon-γ.

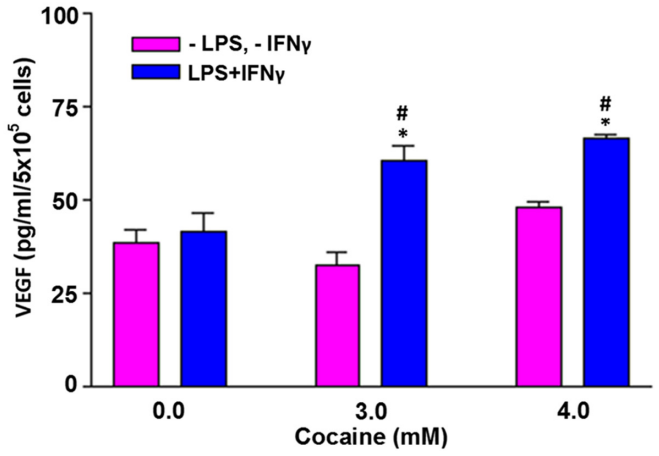

Figure 6. Detection of VEGF using ELISA. Cells in the media lacking phenol red were treated with various concentrations of cocaine in the absence or presence of LPS (0.2 μg/ml) and IFNγ (6 μg/ml) together for 24 h in 96-well culture plates. n=3. *P<0.01 vs. respective 0 mM cocaine with LPS and IFNγ. #P<0.001 stimulated cells treated with 3 or 4 mM cocaine vs. un-stimulated cells treated with 3 or 4 mM cocaine. LPS, lipopolysaccharide; IFNγ, interferon-γ.

VEGF levels are increased by treatment with cocaine in the stimulated cells. In the absence of external challenge, cells treated with cocaine at 3 and 4 mM did not increase VEGF levels (Fig. 6). However, when the cells were challenged with LPS and IFNγ, the VEGF levels in the cells treated with 3 and 4 mM cocaine increased significantly (P<0.05). Compared with the control (41.8±4.5), the average increase was 60.5±3.9 and 66.4±1.1 pg/ml, respectively at 3 and 4 mM cocaine.

Discussion

Consistent with an earlier report (17), cocaine resulted in a dose and time-dependent cell death. Studies have shown that cocaine-induced cellular dysfunction was associated with increased production of reactive oxygen species (20,26) and H_2O_2 (26,27). However, in the present study, cocaine treatment did not increase the production of these oxidative species, which may have been due to either a difference in the cell model used, or a longer period of exposure compared with acute treatment (20).

The ubiquitous biological molecule NO is a small molecule, and a volatile gas with several molecular targets in the body (28). It has both beneficial and harmful effects; for example, at physiological concentrations, it is associated with neurotransmission (29), cognitive function and synaptic plasticity. However, excess production is associated with inflammation with detrimental consequences related to neurological disorders (30,31). Thus, over production of NO represents a diseased-state in the body.

In the present study, a diseased-state was simulated by challenging the astroglia-like cells with endotoxin (LPS), which is commonly found on the cell membrane of gram negative bacteria (32), and a pro-inflammatory cytokine (IFNγ). Using this challenge as a means of activating astrocytes, whether cocaine potentiated the inflammatory response was assessed. Increased NO/NO_2^- production was observed in LPS and IFNγ stimulated cells in the absence of cocaine, indicating a synergistic effect of LPS and IFNγ, an observation consistent with an earlier study in the same cell line (33). Additionally, the further increase in the NO/NO_2^- levels following cocaine treatment suggested that cocaine potentiates NO/NO_2^- production under diseased-states.

Under normoxic conditions, certain enzymes, such as prolyl-hydroxylases domain (PhD) degrade HIF-1α through the proteasomal pathway. This way, the intracellular concentrations of HIF-1α under normoxic conditions are maintained at a low level. However, under a hypoxic environment, the PhD enzymes are inhibited, leading to accumulation of stable HIF-1α in cells. Similarly, a high concentration of NO (>1 μM) also stabilizes HIF-1α under normoxic conditions (34). The presence of stable HIF-1α in the cytoplasm in turn activates several genes required for adaptation under conditions of low oxygen availability (35). One of the more relevant genes targeted by stable HIF-1α is VEGF (36,37). In the present study, detection of HIF-1α and VEGF following cocaine treatment suggested the generation of the hypoxic environment in cells was due to dysfunctional mitochondria owing to a loss of membrane potential (17). The presence of iNOS in astrocytes (9), and production of NO by iNOS under hypoxic conditions (10) triggered HIF-1α and VEGF production in cells (Figs. 5 and 6).

The physiological relevance of the results would have been greater if primary astrocytes were used in the present study; but due to their limited growth potential, finite life span and lack of cell homogeneity between different primary cultures, *C6* astroglia-like cells were instead used. These cells exhibit a high degree of similarity with human astrocytes (16); thus, the results of the present study may also have *in vivo* significance. Several studies have shown the link between inflammation and drug abuse in the past; for example, elevated levels of pro-inflammatory factors were observed in cocaine-dependent individuals (38-41), but no link was established with NO production. Whilst previous reports in non-CNS cells showed cocaine-induced changes in NO levels (42,43), no studies have reported on the effect of cocaine on NO production in astrocytes under a diseased state, to the best of our knowledge.

In conclusion, the present study showed that cocaine potentiated the inflammatory response in LPS and IFNγ-stimulated astroglia-like cells under a diseased-state. Since astrocytes modulate the synaptic cross-talk *in vivo* (7), excess release of NO by activated astrocytes with cocaine could result in dysfunction of neurons and lead to the development or exacerbation of several CNS disorders (44). The involvement of NMDA receptors in excess NO production, their expression in astrocytes (11) and glutamatergic neurons highlights the possibility of using NMDA antagonists as a means of regulating NO levels in the body; which may aid in delaying the onset or progression of several CNS diseases.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

MA, RBB, EM, CBG, and KFS conceived and designed the study, and performed the experiments. RBB, EM and CBG interpreted and analyzed the data and finalized the manuscript. All authors read and approved the final manuscript. RBB, EM, and CBG confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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