Cyanide-Induced Generation of Oxidative Species: Involvement of Nitric Oxide Synthase and Cyclooxygenase-2

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ABSTRACT

In cerebellar granule cells, potassium cyanide (KCN) activates the NMDA receptor resulting in generation of nitric oxide and reactive oxygen species (ROS). To study the mechanism by which KCN stimulates ROS generation, the action of cyanide on the enzymatic pathways known to generate ROS were studied. The oxidant-sensitive fluorescent dye, 2,7-dichlorofluorescin was used to measure intracellular levels of nitric oxide and ROS in cerebellar granule cells. Using selective enzyme inhibitors, it was shown that both protein kinase C and phospholipase A2 are involved in KCN-stimulated generation of NO and ROS. In cells treated with indomethacin or nordihydroguaiaretic acid, inhibitors of cyclooxygenase (COX) and lipooxygenase (LOX) respectively, attenuated (~35%) KCN-induced generation of oxidant species. When L-NAME (L-nitro-L-arginine methyl ester) (nitric oxide synthase inhibitor, NOS) was combined with either indomethacin or nordihydroguaiaretic acid, generation of oxidant species was blocked by more than 80%. Pretreatment with NS398 (COX-2 inhibitor) significantly decreased ROS generation indicating the involvement of COX-2 in KCN-induced oxidant generation. Treatment with L-NAME + NS398 blocked oxidant species generation, reflecting involvement of NOS. The participation of cytochrome P450 was not evident because SKF525A did not significantly reduce KCN-induced ROS generation. Furthermore, a correlation was observed between oxidant generation and lipid peroxidation of cellular membranes (as determined by thiobarbituric acid levels). Pretreatment with inhibitors of protein kinase C, phospholipase A2 or COX, LOX, COX-2 partially blocked KCN-induced formation of thiobarbituric acid reactive substance, whereas coincubation of L-NAME with the inhibitors decreased lipid peroxidation by 60 to 90%. In cytotoxicity studies, KCN-induced cell death was partially blocked by the inhibitors and significant protection was observed when L-NAME was combined with these compounds. These findings show that activation of phospholipase A2 and subsequent metabolism of arachidonic acid by the COX-2 and LOX pathways and NOS contribute to cyanide-induced ROS production.

Cyanide-induced hypoxia is associated with oxidative stress and subsequent peroxidation of lipid membranes in neuronal models (Johnson et al., 1987; Muller and Kriegstein, 1995, Gunasekar et al., 1996). In addition to increased generation of ROS and NO, cyanide inhibits brain antioxidant defense that predisposes to oxidative injury (Ardelt et al., 1989; Gunasekar et al., 1996). As a result, the nervous system is vulnerable to chemical hypoxia-induced cytotoxicity.

Cyanide-induced neurotoxicity is associated with activation of the NMDA subtype of glutamate receptors that initiates a series of reactions leading to oxidative stress. Cyanide influences NMDA receptors both directly and indirectly, leading to destabilization of cytosolic Ca++ homoeostasis (Patel et al., 1994; Sun et al., 1997). Increased cytosolic Ca++ triggers a number of Ca++-dependent pathways, including PLA2 activation that enhances AA metabolism (Lazarewicz et al., 1990; Yang et al., 1994) and Ca++-calmodulin dependent, and PKC regulated nitric oxide generation by NOS (Bredt and Snyder, 1992). In cerebellar granule cells, cyanide produces a Ca++ dependent generation of NO and ROS which is initiated by NMDA receptor activation (Gunasekar et al., 1996). Cyanide can influence NMDA receptor activation by a PKC-sensitive process that is Ca++-dependent. Inhibition of PKC or blockade of the NMDA receptor prevents cyanide-induced cytotoxicity (Rathinavelu et al., 1994; Pavlakovic et al., 1995).

The cyanide response parallels that of glutamate-induced excitotoxicity in which oxidative stress predisposes neurons to injury (Bondy and Lee, 1993). In excitotoxicity, the process underlying enhanced generation of ROS is not clear. Choi

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ABBREVIATIONS: AA, arachidonic acid; CHEL, chelerythrine; COX, cyclooxygenase; COX-2, cyclooxygenase-2; DCF, 2,7-dichlorofluorescin; INDO, indomethacin; LDH, lactate dehydrogenase; L-NAME, N^0-nitro-L-arginine methyl ester; LOX, lipooxygenase; TBARS, thiobarbituric acid reactive substance; NDGA, nordihydroguaiaretic acid; NMDA, N-methyl-D-aspartate; NO, nitric oxide; NS398, N-[2-cyclohexyloxy-4-nitrophenyl]methanesulfonamide; PKC, protein kinase C; PLA2, phospholipase A2; QUIN, quinacrine; ROS, reactive oxygen species; SOD, superoxide dismutase.
(1988) proposed that generation of free radicals during oxidative metabolism of AA is a contributing factor in excitotoxic neuronal injury. AA is a substrate for COX-2 and induction of COX-2 activity can increase generation of ROS producing damage of lipids, proteins and DNA (Nogawa et al., 1997). In the case of cyanide, cyanide-induced activation of phospholipase A2, either as a result of elevated cytosolic Ca$^{2+}$ or membrane lipid damage, can lead to ROS generation (Yang et al., 1994).

Because cyanide neurotoxicity is in part mediated by PKC and PLAD activation, it was proposed that ROS generation may also be related to their activation and subsequent metabolism of AA. In this study, it was determined that inhibition of both NOS and PLAD attenuates cyanide-induced oxidative species and the related cytotoxicity.

**Materials and Methods**

**Chemicals.** DMEM and penicillin/streptomycin solutions were purchased from Gibco (Grand Island, NY); fetal calf serum was from Hyclone (Logan, UT), poly-L-lysine, SOD, catalase, 2-thiobarbituric acid, NADH, pyruvic acid, INDO, NDGA, SKF525A and QUIN were from Sigma Chemical Co. (St. Louis, MO); CHEL was from LC Laboratories (Woburn, MA); DCF-DA was from Molecular Probes Inc. (Eugene, OR); NS398 was from Cayman Chemical Co. (Ann Arbor, MI); L-NAME and MK-801 from RBI (Natick, MA); KCN and KPO$_4$ were from Mallinckrodt (Paris, KY). Thiobarbituric acid assay.

**Quantitation of cytotoxicity.** Cytotoxicity was estimated by measurement of LDH efflux from damaged cells into the medium over 36 hr exposure. Thirty six hr treatment period was selected because minimal cell death was detected at the 12- and 24-hr period. Cerebellar granule cells grown in 6-well culture dishes (10 days in vitro) were used for the assays. All stock solutions of drugs were sterilized by filtration and added in a volume of 10 to 20 µl. All pretreatments were added 5 min before KCN (1 mM). 1 mM KCN was chosen after studying serial dose response to cell death. After 36 hr, medium was removed and cells lysed for 10 min in buffer containing 0.5% v/v Triton X-100 in 0.1 M potassium phosphate buffer, pH 7.4. The buffer was removed after centrifugation at 10,000 rpm for 5 min and LDH activity was determined by the spectrophotometric method of Vassault (1983) in both the medium and lysis buffer. The percent of cellular LDH released was calculated as: % LDH release = LDH in medium/LDH in medium + LDH in lysis buffer. For comparisons, total cellular LDH activity in control cells was 10.9 ± 0.47 U/3 x 10$^6$ cells.

**Statistics.** Data were expressed as mean ± S.E.M. and statistical significance was assessed by one-way analysis of variance followed by Tukey-Kramer multiple range test. Differences were considered as significant at P < .05.

**Results**

**Participation of PKC in KCN-induced oxidative species generation.** We reported earlier that KCN induces generation of NO and ROS following NMDA receptor activation in DCF loaded cerebellar granule cells (Gunasekar et al., 1996). Because inhibition of PKC attenuated cyanide-induced cytotoxicity (Rathinavelu et al., 1994; Pavlakovic et al., 1995), the role of PKC in cyanide-induced oxidative species generation was studied. Cells not exposed to treatment (control) exhibited a minimal increase (20.6 ± 1.3 U) in fluorescence over a 10-min recording period. Chelerythrine, a PKC inhibitor (1 µM), significantly decreased KCN (100 µM) evoked generation of oxidative species by 40%. Coincubation of cells with chelerythrine and SOD (100 U/ml) or catalase (100 U/ml) produced an additional attenuation of oxidative species. Note that we have previously shown that SOD or CAT alone produces no effect on the oxidative fluorescence generated by cyanide (Gunasekar et al., 1996). However, simultaneous pretreatment of cells with L-NAME and chelerythrine did not produce additional attenuation of fluorescence (fig. 1). These results indicate that both PKC and NOS activation are involved in the intracellular oxidation of DCF. It was concluded that generation of NO during cyanide toxicity is regulated by PKC because L-NAME did not produce an additional attenuation of oxidation of DCF when PKC was inhibited.

**Role of PLAD in KCN-induced oxidative species generation.** Figure 2 shows the effect of quinacrine (PLAD inhibitor) on cyanide-stimulated generation of oxidant species.
QUIN reduced KCN-induced fluorescence by 30% and this reduction was enhanced when L-NAME was combined with quinacrine. However, SOD or catalase treatment did not potentiate the effect of QUIN. These results indicate activation of PLA2 plays a primary role in ROS generation, because SOD and catalase did not produce additional attenuation in ROS-mediated oxidation of DCF in the presence of PLA2 inhibitor. It was concluded that AA, the product of PLA2 degradation of membrane lipids, plays a role in generation of ROS. Furthermore, coincubation of cells with inhibitors of PKC and PLA2 produced an additional reduction of oxidant species by >75% (data not shown), indicating both ROS and NO are concurrently produced.

Role of COX/LOX in KCN-induced oxidative species generation. As is shown in figure 3 the KCN-stimulated generation of oxidants was significantly attenuated (>35%) by either INDO (10 µM) or NDGA (50 µM). These compounds inhibit cyclooxygenase (COX) and lipoxygenase (LOX), respectively, enzymes that metabolize AA. It also shows that while NS398 (100 µM), a specific blocker of COX-2, significantly reduced the ROS, SKF525A (50 µM), inhibitor of cytochrome P450, did not significantly attenuate the KCN-induced fluorescence. Additional attenuation of ROS production was noted when L-NAME was combined with either INDO, NDGA or NS398. These results indicate that KCN-induced generation of ROS results in part from activation of COX-2 and LOX pathways.

KCN-induced lipid peroxidation is prevented by PKC, PLA2, COX and LOX inhibitors. Cerebellar granule cells exposed to cyanide (1 mM) for 6 hr exhibited increased TBARS levels. A correlation was seen between NO and ROS formation and lipid peroxidation. TBARS production was partially blocked by pretreating the cells with L-NAME (300 µM), CHEL (1 µM) or QUIN (5 µM) as shown in figure 4A. Figure 4B shows that TBARS production was significantly reduced by pretreating the cells with INDO (10 µM), NDGA (50 µM) or COX-2 blocker, NS398 (100 µM), whereas the cytochrome P450 blocker, SKF525A (50 µM), did not alter TBARS levels. However, KCN-induced lipid peroxidation was further decreased in the presence of L-NAME.

KCN-induced cell death is prevented by PKC, PLA2, COX and LOX inhibitors. In cerebellar granule cells KCN-induced cytotoxicity is mediated by NMDA receptor activation and the cells are partially protected by either L-NAME, SOD or catalase (Gunasekar et al., 1996). KCN-induced cell death was partially blocked by pretreating the cells with L-NAME (300 µM), CHEL (1 µM) or QUIN (5 µM) as shown in figure 4A. Figure 4B shows that TBARS production was significantly reduced by pretreating the cells with INDO (10 µM), NDGA (50 µM) or COX-2 blocker, NS398 (100 µM), whereas the cytochrome P450 blocker, SKF525A (50 µM), did not alter TBARS levels. However, KCN-induced lipid peroxidation was further decreased in the presence of L-NAME.
Discussion

Previous studies have shown that cyanide stimulates simultaneous generation of NO and ROS through a NMDA receptor-mediated process and the subsequent cytotoxicity of cerebellar granular cells is related to NO and ROS production (Akira et al., 1994; Gunasekar et al., 1996). Cyanide-induced generation of NO and ROS is Ca\(^{2+}\) dependent and results from Ca\(^{2+}\) influx via the NMDA channel. Cyanide-stimulated NO production is attributed to NOS activation which is Ca\(^{2+}\)/calmodulin-dependent and the influx of Ca\(^{2+}\) through the NMDA ionophore is sufficient to activate the enzyme (Dawson et al., 1991). In our study it was shown that enhanced generation of NO by KCN in the cerebellar granule cell was blocked by inhibitors of PKC and NOS and it was concluded that PKC is involved in activation of NOS. It was also shown that cyanide-stimulated ROS generation was mediated by Ca\(^{2+}\) activation of PLA\(_2\), leading to AA production and subsequent metabolism by COX and LOX pathways. COX-2 isoenzyme appears to be a major contributor of ROS generation under these conditions. It is proposed that excessive, uncontrolled generation of NO and ROS via these pathways can result in peroxynitrite formation which is a potent oxidant that mediates lipid peroxidation and cytotoxicity (Radi et al., 1991; Gunasekar et al., 1995a).

In neurons, NO is generated after activation of NOS, a Ca\(^{2+}\)/calmodulin-dependent and PKC regulated enzyme (Bredt and Snyder, 1992). NOS activation and overproduction of NO is associated with neuronal cytotoxicity (Dawson et al., 1996). Inhibition of NOS attenuates NMDA-induced...
Ca\textsuperscript{2+} mobilization and the subsequent excitotoxicity in cultured cerebellar granule cells (Mei et al., 1993; Lafon-Cazal et al., 1993). PKC may play an important role in regulating NOS and the subsequent cytotoxicity associated with excessive NOS activity. In several cell models, NO generation is associated with cytotoxicity and inhibition of PKC can decrease the toxicity (Maiese et al., 1993). Down-regulation or inhibition of PKC protects cerebellar granule cells from glutamate toxicity (Favaron et al., 1990; Felipo et al., 1993). However, PKC activation can increase NO production (Severn et al., 1992; Okada, 1995).

The stimulated generation of NO and ROS by KCN in cerebellar granule cells is associated with influx of extracellular Ca\textsuperscript{2+} after NMDA receptor activation (Gunasekar et al., 1996). Activation of NMDA receptors and concomitant Ca\textsuperscript{2+} influx are key events in the neurotoxic response (Patel et al., 1993, 1994; Sun et al., 1997). Our study showed that activation of NOS during cyanide toxicity is partially regulated by PKC. Our previous studies have shown that cyаниde-induced PKC activation and subsequent cytotoxicity are prevented by both NMDA receptor antagonists and NOS inhibitors (Rathinavelu et al., 1994; Pavlakovic et al., 1995), providing evidence for involvement of PKC in the activation of NOS during cyanide toxicity. It is concluded that during cyanide toxicity PKC activation plays a critical role in the generation of NO by regulating NOS activity.

In cerebellar granule cells, increased cytosolic Ca\textsuperscript{2+} and subsequent production of AA via PLA\textsubscript{2} activation can lead to ROS generation (Miller et al., 1992; Oyama et al., 1994; Tang et al., 1996). PLA\textsubscript{2} activation is associated with O\textsuperscript{2-} under a variety of conditions (Miller et al., 1992; Lafon-Casal et al., 1993) and enhanced generation of ROS during oxidative metabolism of AA to eicosanoids has been suggested to be involved in the excitotoxic neuronal injury (Choi, 1988). Receptor-mediated generation of ROS is dependent on influx of extracellular Ca\textsuperscript{2+} and subsequent PLA\textsubscript{2} activation (Miller et al., 1992). PKC may also play a role in PLA\textsubscript{2} activation and AA generation. In PC12 cells, PKC inhibitors can alter PLA\textsubscript{2} activity and decrease AA release from the cells (Zheng et al., 1996).

In our study, quinacrine (a PLA\textsubscript{2} inhibitor) partly attenuated cyaniide-induced oxidant species generation and lipid peroxidation. These observations are consistent with another report in which inhibition of PLA\textsubscript{2} or lipid peroxidation partly protected cerebellar granule cells against cyaniide toxicity (Müller and Kriegstein, 1995). Activation of PLA\textsubscript{2} by elevated cytosolic Ca\textsuperscript{2+} or damage to cellular membranes by cyaniide has been reported in PC12 cells (Yang et al., 1994). Based on in vitro experiments, it has been reported that the NMDA-induced increase of O\textsuperscript{2-} was suppressed by PLA\textsubscript{2} inhibition (Fagni et al., 1994; Gunasekar et al., 1995a), consistent with the proposal that ROS generation results from PLA\textsubscript{2} activation and subsequent AA metabolism. Alternatively, it is possible that cyaniide-induced mitochondrial dysfunction can also lead to ROS generation (van de Water et al., 1994).

In neuronal models PLA\textsubscript{2} activation increases the susceptibility of membrane phospholipids to hydrolytic processes that are associated with AA production (Shimizu and Wolfe, 1990). AA can be converted to the biologically active metabolites, prostaglandins and hydroperoxyeicosatetraenoic acid (HPETE) by COX and LOX pathways, which is accompanied by O\textsuperscript{2-} production. Neuronal cells produce COX and LOX products of AA metabolism under a variety of conditions (Bishai and Coccane, 1992). Rothman et al. (1993) and Lerea et al. (1995) suggested that AA metabolism is activated by NMDA and other excitotoxic amino acids. The involvement of COX and LOX metabolites in O\textsuperscript{2-} generation has been studied in macrophages and other cells by using specific COX and LOX inhibitors (Phillis, 1994; Mayer et al., 1995). In our study, by use of COX and LOX inhibitors, the two pathways of AA metabolism were shown to play a significant role in KCN-induced ROS formation because the inhibitors attenuated ROS production and related membrane peroxidation. These findings parallel previous observations showing that COX and LOX blockers can attenuate neurotoxicity-induced by NMDA and kainate (Rothman et al., 1993; Phillis et al., 1994; Lerea et al., 1995; Hewett et al., 1996). Furthermore, the involvement of cytochrome P450 in KCN-induced ROS is not evident because inhibition of cytochrome P450 did not significantly attenuate the KCN-induced ROS and cytotoxicity.

The involvement of COX-2 activation in KCN-induced ROS was determined because recent reports suggest COX-2 upregulation during cerebral ischemia is associated with enhanced production of free radicals and postischemic prostaglandin accumulation (Adams et al., 1996; Nagawa et al., 1997). In the normal brain COX-2 is expressed in selected neurons where it can be induced and upregulated during high frequency stimulation and after seizures that can be prevented by treatment with the NMDA receptor antagonist MK801 (Yamagata et al., 1993). It was observed in this study that pretreatment of granule cells with NS398, a COX-2 inhibitor, attenuated generation of ROS induced by KCN. These results provide strong evidence that the COX-2 pathway is an important route for AA metabolism and oxidative species production during cyanide exposure.

The failure of either COX or LOX inhibitors to afford complete protection to the granule cells can be explained by involvement of multiple processes in the cytotoxic response. Protection against the cytotoxicity was observed when the cells are pretreated with either COX or LOX inhibitors combined with the NOS inhibitor L-NAME. Concurrent generation of NO and ROS by different pathways appear to be stimulated by cyaniide. Overproduction of ROS and NO would lead to peroxynitrite formation and then oxidative cell injury (Gunasekar et al., 1995a). Also it is interesting to note that cyaniide inhibits the brain antioxidant defense (catalase, superoxide dismutase and glutathione peroxidase) which would predispose to oxidative injury (Ardelt et al., 1989).

In conclusion, this study demonstrated that cyaniide-induced generation of NO is mediated by the activation of PKC regulated NOS in cerebellar granule cells. ROS generation was related to PLA\textsubscript{2}-mediated production of AA, followed by metabolism via the cyclooxygenase and lipoxygenase pathways. These findings also provide evidence that COX-2 isoenzyme contributes to KCN-induced ROS. Concurrent inhibition of COX/LOX and NOS protects against cyaniide induced lipid peroxidation and cell death.

References


