REPORT

Role of prostaglandin H synthase in activation of acrylonitrile to cyanide

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Abstract: The present study aimed at investigating the in-vitro oxidation of acrylonitrile (ACN) to cyanide (CN^-) by prostaglandin H synthase (PHS). Detection of CN^- is considered a marker for free radical intermediates involved in ACN-induced toxicity. First, most favorable circumstances for ACN oxidation were characterized: pH (4.5), temperature (37ºC) and time of incubation (60 min.). In addition, the concentrations of ACN, PHS and H_2O_2 in incubation mixtures were assessed for further reaction characterization. The reaction maximum velocity (V_{max}) was calculated to be 582.75 pmol CN^-/mL/min and the Michaelis-Menten constant (K_m) was 149.25 µmol ACN. Adding PHS inhibitors; resveratrol, quercetin, indomethacin or troloc-C to the reaction mixtures significantly reduced the rate of ACN oxidation. In conclusion, the present study demonstrates the ability of PHS to oxidize ACN to CN^- and provides a clue for the explanation of ACN target toxicity.

Keywords: Acrylonitrile, prostaglandin H synthase, cyanide.

INTRODUCTION

Extrahepatic xenobiotic metabolism is becoming in the center of interest for elucidating target specificity of environmental chemicals (Gundert-Remy *et al.,* 2014). Prostaglandin H Synthase (PHS) has gained interest as a drug metabolizing enzyme (Tsai and Kulmacz, 2010). The concurrent reactions during arachidonic acid (AA) metabolism depend on PHS peroxidase activity. For non-cosubstrate chemicals, their epoxidation relies on the subsequent formation of peroxyl radicals. A lot of chemicals are metabolized via chemical reactions involving free radical (FR) mechanisms. A growing body of evidence indicates the contribution of these FR-mediated reactions in toxic metabolites formation, in addition to the detoxification of chemicals. In order for this type of metabolism to occur, the prevailing factor is the relative concentrations of PHS and peroxyl radicals with reference to other systems, particularly the monooxygenase system. The role of peroxidase and peroxyl radical systems in both activation and detoxification of chemicals in extrahepatic tissues is strongly supported by in-vivo investigations (Dekant, 2009).

Acrylonitrile (ACN) has been largely investigated for its toxicity; however, few studies could explain its harmful mechanism. To induce cancer, ACN has to be metabolically activated (Pu *et al.,* 2006). Interestingly, CYP 2E1 - the main ACN metabolizing enzyme - is highly abundant intrahepatically. Yet, liver is not a specific target of ACN-induced carcinogenicity (Wang *et al.,* 2002). ACN is suggested to be activated *in-situ*, by local enzymes at the target organs. The current study investigated this suggestion, using PHS enzyme expressed in GIT, testes, and prostate, for its capability to metabolize ACN to CN^-. The detection of CN^- is considered as a marker of ACN metabolism throughout a pathway involving FR intermediates (Abdel-Naim and Mohamad in, 2004; Mohamadin and Abdel-Naim, 2003). These FRs were particularly involved in oxidative stress and tumor formation. Therefore, the current study aimed at investigating the ability of PHS enzyme system to *in-vitro* metabolizes ACN to CN^-.

MATERIALS AND METHODS

Chemicals

ACN, quartering dehydrate and resveratrol were purchased from Acros Organics (Geel, Belgium). Indomethacin, potassium dicyanoargentate and PHS were bought from Sigma Aldrich Chemie (Steinheim, Germany). Potassium cyanide, hydrogen peroxide, disodium hydrogen orthophosphate dodecahydrate, sulfuric acid and sodium hydroxide were purchased from Fisher Scientific (Loughborough, Leicestershire, UK).

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All other chemicals were of highest purity commercially available.

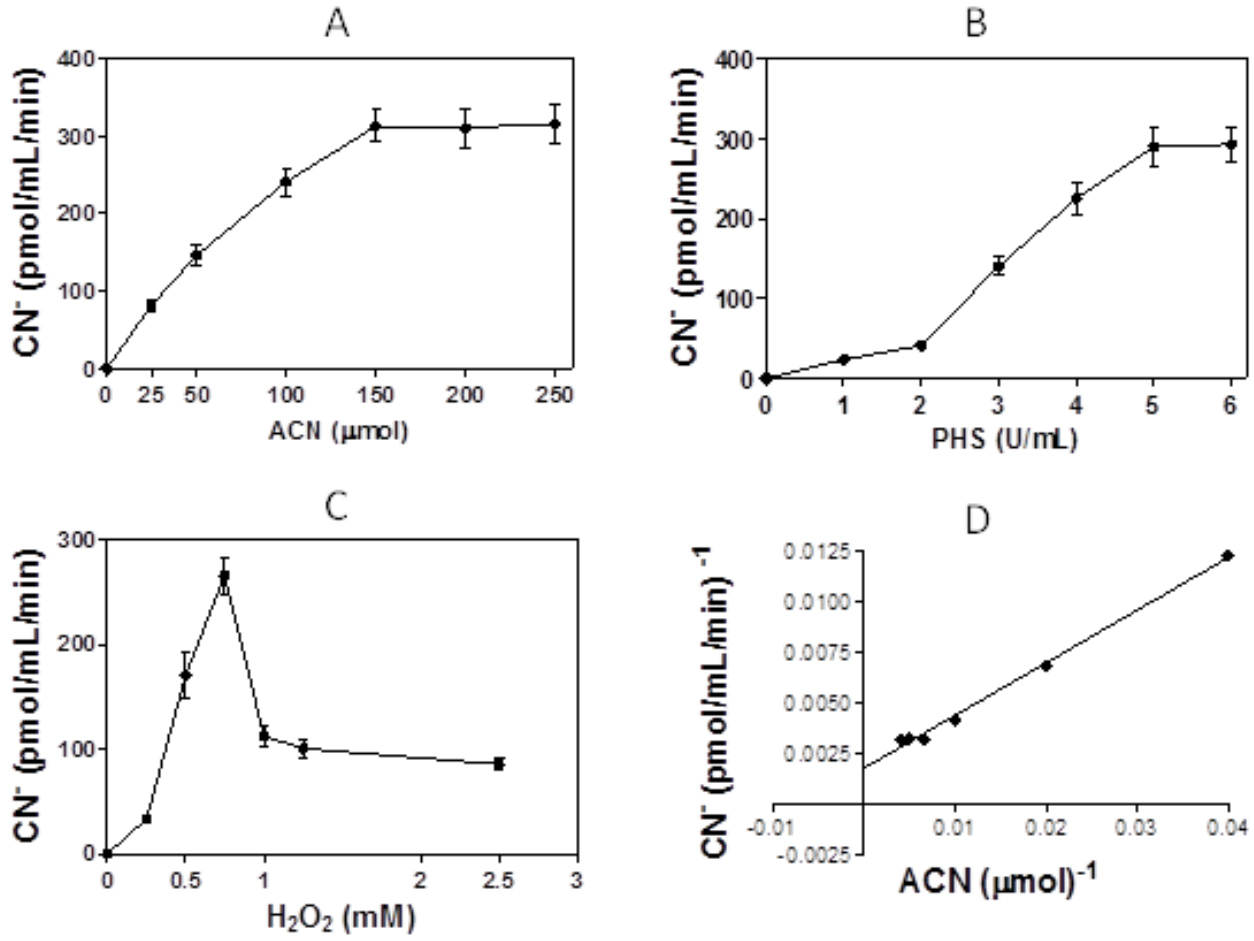
Cyanide assay

The *in-vitro* assay of CN^- was conducted according to the method of Abreu and Ahmed (1980). Exactly, 2mL of 0.1M NaOH was put in the central compartment and 2mL of 4.0N H_2SO_4 was added in the outer compartment of the Conway micro diffusion plates, purchased from Science ware Bel Art Products (Pequannock, NJ, USA). Then, triplicate dilutions of incubation mixtures (2mL each) were added to the outer compartment with H_2SO_4 and instantaneously preserved with greased covers to prevent any thrashing of gases generated by the reaction. Thereafter, plates were shaken at 70 rpm for 1 hour.

Cyanide was formed as HCN as a result of reactions in the outer compartment, followed by recovery into the central compartment. After the due time, the plates were uncovered and the outer compartment contents were sucked. The indicator $KAg(CN)_2$ (50 µL) was put in the central compartment while mixing. This indicator solution was composed of 33g ($Na_2HPO_4 \cdot 12H_2O$), 2.2g (NaOH) as well as 10mg KAg $(CN)_2$ in 100 mL water. Both the selective electrode "Orion Silver/Sulfide Ion" and the reference electrode "Orion Double Junction Silver/Silver chloride" – obtained from Thermo Electron Corporation, Auchtermuchty, Fife, Scotland - were placed into the central compartment. Thereafter, they were connected to a Jenway Electric Digital pH/mV meter (model 3310). The potential (mV) reading was recorded for each plate which is due to release of free silver ions by CN^- ensnared in the central compartment of the Conway micro diffusion plate.

Incubation mixtures

The incubation mixture was formed of 5U/mL PHS, 0.75 mM H_2O_2, 150 µmol ACN completed to 2.2mL via 0.1M phosphate buffer (pH 4.5). The reaction was begun by adding H_2O_2. The mixtures were packed into scintillation vials and incubated at 37°C for 15 min in a shaking water bath. Then, the vials were engrossed into ice to make certain that the reaction was shut-down. Each reaction



Data are presented as mean ± S.E. of six replicate determinations.

Fig. 1: Effect of ACN concentration (A), PHS concentration (B), and H_2O_2 concentration (C) on the metabolism of ACN to CN^-. Kinetics of ACN metabolism to CN^- by PHS/H_2O_2 system (D).

mixture (2mL) was put in the outer compartment of the Conway microdiffusion plate as previously mentioned. The potential readings in mV were utilized to calculate the concentration of CN^- as well as the rate of CN^- formation in pmol/mL/min after constructing a standard calibration curve.

Table 1: Effect of resveratrol, quercetin, indomethacin, and troloc-C on the metabolism of ACN to CN^-

Reaction Condition	CN^- pmol/mL/min)
Control (reaction mixture only)	289.97±16.5
Control + Resveratrol 0.2mM	216. 31*±20.2
Control + Quercetin 0.2mM	231.30*±11.6
Control + Indomethacin 0.2mM	137.25*±8.2
Control + Trolox-C 0.2mM	127.46*±12.2

Data are presented as mean ±S.E. of six replicate determinations. Each reaction mixture contained 150 µmol ACN, 5U/mL PHS, and 0.75mM H_2O_2. *Significantly different from control ($p<0.05$)

Data analysis

All data were presented as the mean values ± S.E. of six independent experiments. Graphs were sketched using GraphPad Prism software version 5 (GraphPad Software, Inc., La Jolla, CA, USA). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test, by means of GraphPad Instat 3 software (GraphPad Software, La Jolla, CA, USA). The 0.05 level of probability was used as the criterion for significance. The kinetics parameters, maximum velocity (V_{max}) and Michaelis-Menten constant (K_m), were obtained by Lineweaver-Burk plot.

RESULTS

The characterization of ACN activation into CN^- via the PHS enzyme system was carried out based on the reaction conditions and reactant stoichiometry. The enzymatic activity of PHS was maintained at temperature range (25 - 65ºC). The maximum rate of CN^- generation was detected at 37ºC. This was achieved at optimal pH 4.5. Moreover, the rate of CN^- production was directly related to incubation times until 60 min, and then the reaction showed a discernible decline (fig. are included as supplementary data).

Concerning reactants stoichiometry, the rate of CN^- generation from a series of ACN concentrations was concentration-related till 150 µmol, then no more boosting of CN^- formation was detected (fig. 1A). Regarding PHS concentration, the current findings have shown that increasing PHS concentration from 2 to 5 U/mL resulted in an apparent rise of CN^- release. The maximal rate of CN^- formation was found at PHS concentration (5U/mL). No additional increase in CN^- production was observed with higher PHS concentrations (fig.1B). Furthermore, CN^- production rate - by PHS - was directly related to H_2O_2 concentration. Though, at concentrations above 0.75mM H_2O_2, the rate of CN^- formation was declined (fig. 1C). According to these experimental conditions, further incubations were carried out at pH (4.5), a temperature (37ºC), and an incubation period (60 min). Besides, 150 µmol ACN, 5 U/mL PHS, and 0.75 mM H_2O_2 were selected for the incubation mixtures. Under the previously-indicated conditions, the reaction constants controlling the rate of ACN metabolism by the PHS/H_2O_2 system were assessed. V_{max} - the reaction maximum velocity - was found to be 582.75 pmol CN^-/mL/min as well as the Michaelis–Menten constant (K_m) was 149.25 µmol ACN (fig. 1D).

Several inhibitors of the PHS/H_2O_2 system were tested at 0.2mM concentration (table 1). Co-incubation of resveratrol and quercetin with PHS/H_2O_2 system caused a significant reduction of the rate of CN^- formation by 25.3% and 20.0%, respectively, as compared to the control. Indomethacin incubation had caused a higher and significant decrease in the rate of CN^- release by 52.6% of the control. Addition of the trolox-C to incubation mixtures resulted in 56.0% decrease in CN^- release.

DISCUSSION

ACN has been thoroughly investigated for its toxic effects. In order to cause cancer, previous studies have focused on the important role of ACN metabolic activation (Pu *et al.,* 2006). It is worthy noted that CYP 2E1 resides mainly intrahepatically (Wang *et al.,* 2002). Despite this fact, it is not targeted by ACN for carcinogenicity, which raised the attention that ACN could be activated *in-situ*, by local enzymes its target organs of carcinogenicity. Therefore, the current study aimed to investigate the capability of PHS enzyme system to metabolize ACN to CN^- *in-vitro*.

The formation of CN^- requires ACN oxidation, resulting in formation of a reactive and a quite long-lasting epoxide named 2-cyanoethylene oxide (CEO) (Kedderis *et al.,* 1995). Further metabolism of CEO can release CN^- (Hadri *et al.,* 2005; Wang *et al.,* 2002). It is worth noted that the detection of CN^- was a marker of metabolism via hydroxyl free radicals generated in a Fenton-like reaction (Mohamadin, 2001). ACN is well-known to induce oxidative stress (Al-Abbasi, 2012; Caito *et al.,* 2014).

Oxidative stress is the result of an imbalance that exists between the reactive oxygen species (ROS) and antioxidant defense mechanisms in a tissue (Esmat *et al.,* 2007). Upon their generation, ROS interact with and alter both structural and functional macromolecules in the cell. This may result in the modification of cell function through oxidative damage to lipids (lipid peroxidation), DNA or proteins which may even result in cell death (Messens *et al.,* 2015). Further metabolism of CEO into CN^- was reported to provoke oxidative stress via the

generation of ROS and nitric oxide (Gunasekar *et al.,* 1996).

The PHS/H_2O_2 enzyme system was characterized with respect to pH, temperature, and time of incubation mixtures. It has been shown that PHS/H_2O_2 system can maximally oxidize ACN to CN^- at a temperature of 37 °C and a pH of 4.5. Thus, acidic pH was chosen for further experimentation. Chronic exposure of rats to ACN has been linked to inflammatory responses, causing a decrease in pH to below physiological value (Quast, 2002). Therefore, nitriles-induced inflammation would lower pH leading to higher rates of its own biotransformation. Concerning the incubation time, maximum rate of CN^- formation was detected after 60 min.

In addition, the maximum CN^- formation was detected in mixtures having H_2O_2 (0.75mM). Higher concentrations of H_2O_2 resulted in reduced rates of CN^- release. Based on our experimental conditions, assay conditions were chosen to be: a pH of 4.5, a temperature of 37°C, an incubation time of 60min, ACN concentration of 150 µmol, a PHS amount of 5U/mL and H_2O_2 concentration of 0.75mM. These data noticeably reveal the ability of the PHS/H_2O_2 system to oxidize ACN into CN^-. To further typify the reaction, PHS inhibitors like resveratrol, quercetin, indomethacin and trolox-C were assessed. Both resveratrol and quercetin could significantly hold back CN^- formation. Resveratrol (trans-3, 5, 4'-trihydroxystilbene) is a polyphenolic compound found in many members of plant kingdom. It has several pharmacological actions, including anti-inflammatory properties. Recently, it has been assessed for its potential protective role in neurodegenerative diseases. Resveratrol decreases the expression of many inflammatory mediators implicated in the progression of neuropathological conditions. In addition, it has been shown to provide neuronal protection in different models (Wendeburg *et al.,* 2009). It has been shown that quercetin acts as an antioxidant *in-vitro* (Filipe *et al.,* 2004) and inhibits the nitric oxide pathway (El-Shafey *et al.,* 2015). It also has anti-inflammatory activity by targeting proinflammatory signaling pathways, such as STAT1, NFκB and MAPK (Hamalainen *et al.,* 2007).

Furthermore, non-steroidal anti-inflammatory drugs like indomethacin more significantly inhibited PHS activity. This was in agreement with previous studies showing that indomethacin inhibited other peroxidases such as myeloperoxidase (Abdel-Naim and Mohamadin, 2004). Additionally, indomethacin-lipoperoxidase interaction had been previously characterized by Chatterjee et al. (1996). The addition of trolox C - a water soluble analog of vitamin E - to lipoperoxidase system was associated with a substantial fall in the rate of CN^- formation (Nasralla *et al.,* 2009).

It is worthy mentioned that the *in-vivo* generation of CN^- through PHS/H_2O_2 system-mediated pathway may be inconsequential when compared to the yield of CN^- via the hepatic metabolism of CAN (Lipscomb *et al.,* 2009). This pathway represents an alternative source of oxidative stress in organs lacking CYP 2E1.

CONCLUSION

The current study demonstrates the ability of PHS/H_2O_2 enzyme system to oxidize ACN to CN^- *in vitro* and provide insight for clarification of ACN chronic toxicity.

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