Antioxidant properties of argpyrimidine

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ABSTRACT

Argpyrimidine, the product of non-enzymatic protein glycation by methylglyoxal, has been implicated in the pathophysiology of diabetes mellitus and neurodegenerative diseases. Chemically, argpyrimidine is a substituted pyrimidinol with structural features common to known antioxidants. The objective of this study was to investigate the antioxidant properties of argpyrimidine. Argpyrimidine was synthesized by mixing L-arginine with 3-acetoxypentane-2,4-dione under acidic conditions and purified by chromatography. Argpyrimidine inhibited lipid peroxidation of rat brain homogenates catalyzed by hydroxyl radicals, metal ions, and autoxidation in a concentration- and time-dependent manner. In addition, argpyrimidine scavenged superoxide anion, 1,1-diphenyl-2-picryl-hydrazyl-stable free radical, intracellular-hydrogen peroxide, and inhibited free-radical-mediated nicking of plasmid-DNA. Taken together, our data suggest that argpyrimidine has antioxidant properties and may therefore have biological relevance in pathophysiologies associated with diabetes mellitus and neurodegenerative diseases.

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1. Introduction

Glycation of proteins comprises a series of non-enzymatic reactions between glucose and proteins referred to as the Millard reaction (Thornalley, 1999). The protein adducts formed via the Millard reaction are collectively termed as advanced glycation end products. These advanced glycation end products have been implicated in the long term complications of diabetes mellitus such as atherosclerosis, retinopathy, nephropathy, and cataract (Brownlee, 2000). Advanced glycation end products are also thought to be involved in the process of aging (Shibata et al., 2001) and neurodegenerative diseases (Kikuchi et al., 1999).

Methylglyoxal, formed endogenously in all living cells, is a potent glycating agent for intracellular proteins (Richard, 1993). The dicarbonyl moieties of methylglyoxal react irreversibly with amino groups of proteins, resulting in the formation of advanced glycated end products (Booth et al., 1997; Thornalley, 1999). Although methylglyoxal reacts with the amino group of lysine and guanidino group of arginine, under physiological conditions, the reaction with arginine predominates (Ahmed et al., 2005). Chemically, two molecules of methylglyoxal react non-enzymatically with arginyl residues on protein to form argpyrimidine [N-(5-hydroxy-4,6-dimethylpyrimidin-2-yl)-l-ornithine] (Fig. 1) (Shipanova et al., 1997).

The existence of specific cellular receptors for glycated arginine residues (Westwood et al., 1997) suggests that these residues may be of biological relevance. Argpyrimidine has been detected in renal tissues (Oya et al., 1999; Padival et al., 2003) and in lens proteins (Ahmed et al., 1997; Wilker et al., 2001) from diabetic patients. Recent studies have demonstrated the presence of argpyrimidine in amyloid proteins of subjects with familial amyloidotic polynearopathy and in human tumors (Gomes et al., 2005; van Heijst et al., 2005). In experimental animals, the accumulation of argpyrimidine has been demonstrated in the cerebral artery following reperfusion injury (Oya et al., 1999). Thus by “guilt of association”, argpyrimidine has been implicated as a potential cause and/or consequence in the pathophysiology of diabetes mellitus and related diseases.

Chemically, argpyrimidine is a pyrimidinol with electron-releasing methyl group substituents at positions 4 and 6 on the pyrimidine ring, flanking the hydroxyl group (Fig. 1). The antioxidant properties of α-tocopherol, a major biological antioxidant, are attributed to its phenolic structure with electron-donating substituents at the ortho- and para-positions of the phenolic-hydroxyl group. Similarly, butylated-hydroxy toluene (BHT), another potent chain-breaking antioxidant, is also a hindered-phenol with tert-butyl groups flanking the phenolic-hydroxyl group. Both alpha-tocopherol and butylated-hydroxy toluene mediate their antioxidant actions by transferring their phenolic hydrogen atom to “lipid radicals,” thus inhibiting the free radical-mediated propagation of lipid peroxidation. In addition, the electron-releasing substituents flanking the phenolic group weaken the O=H bond by preferential stabilization of phenoxyl and/or destabilizing the phenol moiety (Lucarini et al., 1996). Since these antioxidants inhibit the propagation of the chain-reaction of lipid peroxidation they are often referred to as ‘chain-breaking antioxidants’ (Valignigil et al., 1999).

It has been recently suggested that 5-pyrimidinol with electron-donating substituents could function as effective H-atom donors similar to phenols, with an added advantage of being more resistant to
autoxidation (Pratt et al., 2001). Given this background, we hypothesized that argpyrimidine, a hindered 5-pyrimidinol with an arginine moiety at position 2 (which would further contribute to destabilizing the hydroxyl bond), will function as a potent free radical scavenger. Therefore, in the present study, we investigated the ability of argpyrimidine to attenuate oxidant-stress under in-vitro conditions.

2. Materials and methods

2.1. Materials

SH-SY5Y neuroblastoma cell line (CRL-2266) was obtained from ATCC (Manassas, VA). Minimum essential medium, penicillin-streptomycin solution, newborn calf serum, and trypsin-EDTA were obtained from Invitrogen Corporation (Carlsbad, CA). 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate dicarboxy methyl ester (H₂-DCFDA) was obtained from Molecular Probes (Engene, OR). All other reagents and chemicals were supplied by Sigma-Aldrich Chemical Co (St. Louis, MO).

2.2. Synthesis and characterization of argpyrimidine

Argpyrimidine was synthesized as reported previously (Cavill and Solomon, 1955; Shipanova et al., 1997) with minor modifications. The scheme of synthesis is illustrated in Fig. 2. Briefly, L-arginine (208 mg, 1.2 mmol) was dissolved in concentrated HCl (9.0 ml). To this solution, 3-acetoxypentane-2,4-dione (222 mg, 1.4 mmol) was added, and the mixture was stirred at room temperature for 7 h. To this solution, 3-acetoxypentane-2,4-dione (222 mg, 1.4 mmol) was added, and the stirring was continued for 14 h, following which a final portion of 3-acetoxypentane-2,4-dione (222 mg, 1.4 mmol) was added and stirred for another 7 h. The reaction mixture was cooled to 0°C on an ice bath, and 40 ml of deionized water was added dropwise. The ice-cold solution was extracted with three 50 ml portions of diethyl ether, and the ether layers were discarded. The remaining aqueous phase was evaporated to obtain 414 mg of dark tan foam, which was purified by medium pressure liquid chromatography utilizing a C₁₈ reverse phase packing. The material in a tubing loop was very slowly loaded on the column at a 0.5 ml/min flow rate. After 31 min, a 50:50 acetonitrile:water (0.1% TFA) solution, and the column was eluted at a flow rate of 1.0 ml/min using the following solvent gradient: 1:99 acetonitrile:water (0.1% TFA) from 0–20 min followed by a gradient from 20–45 min ending at 50:50 acetonitrile:water (0.1% TFA). The t₀ was 28.5 min with a determination of 100% purity by fluorescence detection, 100% purity by UV detection at 320 nm and 93% purity by UV detection at 210 nm. Positive ion electrospray ionization mass spectroscopy: Calculated: C₁₁H₂₀O₄N₄ m/z (M+H) 255.1. Found: C₁₁H₁₉O₄N₄ m/z (M+H) 255.1.

2.3. Lipid peroxidation assay

The extent of lipid peroxidation was assessed by measuring the malondialdehyde formation as reported by us previously (Sreejayan and von Ritter, 1998). Briefly, fresh rat brain (50 mg of cerebral cortex) was homogenized in 1 ml of ice-cold TBS buffer (pH 7.4, 20 mM) pretreated with Chelex-100 resin to remove any possible metal contamination. Homogenates were incubated with ascorbic acid (1 mM) and hydrogen peroxide (1 mM) for 30 min in the absence or presence of argpyrimidine (0.1–1.0 mM). To assess the effect of argpyrimidine on the rate of lipid-peroxide formation, argpyrimidine (1 mM) was added to the reaction mixture at different time points, the reaction was stopped, and the extent of lipid peroxidation was analyzed. For metal ion-catalyzed peroxidation, homogenates were stimulated with ascorbic acid (1 mM) and ferric chloride (0.1 mM) for 30 min at room temperature. Peroxidation induced by autoxidation was assessed by allowing the homogenate to undergo spontaneous oxidation in the presence of air at 37°C. Brain homogenates incubated at 4°C were used as controls for such experiments. In all the above cases, lipid peroxidation was assessed as follows: the reaction was stopped by adding 1 ml of ice-cold HCl (0.25 N) containing trichloroacetic acid (10%), thiobarbituric acid (0.38%), and butylated hydroxytoluene (0.05%). Butylated hydroxytoluene was added to the reaction to avoid any artifacts that could arise due to the generation of lipid peroxides during the heating stage of the experiment. Following heating at 80°C for 15 min, samples were cooled and centrifuged, and the pink-colored malondialdehyde (MDA) adduct of thiobarbituric acid in the supernatant was estimated by measuring the absorbance at 532 nm. The amount of lipid peroxidation was determined using the molar extinction coefficient of MDA, 1.56 × 10⁴ M⁻¹ cm⁻¹, and expressed as nmoles MDA per mg protein. Proteins in aliquots of the homogenates were determined by the bicinchoninic acid method.

2.4. Plasmid-DNA nicking assay

The ability of argpyrimidine to protect against oxidative damage to DNA was assessed by using a plasmid-DNA nicking assay (Yang et al.,...
2.5. Interaction of argpyrimidine and 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) stable free radical

DPPH reduction was assessed by incubating a methanol solution of DPPH (final concentration 0.1 M) with an aqueous solution of argpyrimidine for 20 min at room temperature and measuring the absorbance at 517 nm (Sreejayan and Rao, 1996).

2.6. Reduction of ferric ion by argpyrimidine

Aqueous solutions of ferric chloride (0.1 mM) and 1, 10-phenanthroline (0.3 mM) in methanol were incubated with argpyrimidine (0.01–1 mM) for 2 min at room temperature, and absorbance was read at 508 nm (Coward et al., 1993; Firuzi et al., 2005). A standard curve obtained by treating ferrous ion with 1,10-phenanthroline was used to calculate the amount of ferrous ions formed.

2.7. Cell culture and fluorescence determination

Human neuroblastoma (SH-SY5Y) cells were propagated in a minimum essential medium with fetal bovine serum (10%) and penicillin and streptomycin (50 U/ml each) in an incubator at 37 °C in a minimum essential medium with fetal bovine serum (10%) and diacetate (H2-DCFDA) as reported previously (Wang and Joseph, 1999). Generation of intracellular reactive oxygen species was assessed by the fluorescent dye chloromethyl-2,7-dihydrofluorescein diacetate (H2-DCFDA) as reported previously (Wang and Joseph, 1999).

2.8. Superoxide scavenging capacity

Xanthine oxidase (0.07 U/ml), xanthine (0.1 mM), argpyrimidine (0.1–1.0 mM) and nitroblue tetrazolium (0.6 mM) in phosphate buffer (pH 7.4, 0.1 M) were incubated at 25 °C for 10 min, and the absorbance of the formazan formed was read at 560 nm (Sreejayan and Rao, 1996). The direct effect of argpyrimidine on xanthine oxidase activity was evaluated by measuring the formation of uric acid (absorbance maximum 295 nm) by incubating argpyrimidine (0.1–1.0 mM) and xanthine oxidase (0.05 U/ml).

2.9. Data analysis

Each treatment included 3–4 independent determinations run in triplicates. Data are expressed as mean±S.E.M and statistically evaluated using Student’s paired t-test using Sigma Plot statistical software (Jandel Scientific, San Rafael, CA). A P value of less than 0.05 was considered statistically significant.

3. Results

3.1. Argpyrimidine inhibits lipid peroxidation induced by hydrogen peroxide and ascorbic acid

Hydrogen peroxide in the presence of ascorbic acid induces the formation of hydroxyl radicals that can initiate the chain process of lipid peroxidation (Niki et al., 2005). As expected, treatment of brain homogenates with hydrogen peroxide and ascorbate caused a robust (ten-fold) increase in lipid peroxidation compared to the control (1.8±0.14 nmol MDA/mg protein versus 19.2±1.5 nmol MDA/mg protein respectively) that was inhibited by argpyrimidine in a concentration- and time-dependent manner (Fig. 3A and B respectively). As seen in Fig. 3B, the inhibition of lipid peroxidation by argpyrimidine was evident from the very early stages of its induction.

3.2. Argpyrimidine inhibits metal-catalyzed lipid peroxidation and autoxidation of lipids

Transition metal ions such as iron ions can undergo single electron redox reactions and therefore are excellent candidates for abstraction of electrons from electron-rich polysaturated fatty acids, causing initiation of the chain-reaction of lipid peroxidation (Niki et al., 2005). As expected, ferric ions and ascorbate-induced lipid peroxidation (1.9±0.17 to 14.1±0.05 nmol MDA/mg protein) and treatment with argpyrimidine (1 mM) caused a 50% inhibition of metal-catalyzed peroxidation (Fig. 4A). Exposure to atmospheric oxygen at 37 °C for 2 h caused a four-fold increase in lipid peroxidation compared to the homogenates stored at 4 °C (Fig. 4B) that were inhibited by argpyrimidine (Fig. 4B). In both these models, argpyrimidine exhibited both concentration- and time-dependent inhibition of peroxidation (data not shown).

3.3. Argpyrimidine inhibits DNA-cleavage under physiologically relevant conditions

Induction of single strand breaks in supercoiled DNA has been used as a surrogate marker for oxidative damage (Soh et al., 2003). The ability of argpyrimidine to inhibit free radical-induced damage to pUC19 DNA was monitored by observing the conversion of the supercoiled plasmid-DNA (faster migrating species, 1kb) to the circular, nicked form (slower migrating species, 3kb). Ascorbic acid and hydrogen peroxide caused nicks in plasmid-DNA, which was inhibited by argpyrimidine (0.1–1 mM) in a concentration-dependent manner (Fig. 5A and B).
3.4. Argpyrimidine reduces DPPH-stable free radical

DPPH is a stable free radical, and its reduction has been used for the determination of efficacy of antioxidant compounds (Aaby et al., 2004; Litvinenko and Ingold, 2003; Sreejayan and Rao, 1996). Fig. 6A shows that argpyrimidine interacts strongly with DPPH, and at a concentration of 0.1 mM it completely reduces all the DPPH-radicals present.

3.5. Argpyrimidine reduces ferric to ferrous ions

Data presented in Fig. 6B show that argpyrimidine can reduce ferric ions to ferrous ions in a concentration-dependent manner. At equimolar concentrations (0.1 mM), an almost complete reduction of ferric ions was achieved by argpyrimidine.

3.6. Argpyrimidine scavenges hydrogen peroxide in intact cells

DCFDA is a fluorescent probe that passively diffuses into cells where its acetate groups are cleaved by intracellular esterases. Subsequent oxidation of DCF by free radicals yields fluorescent adduct that is trapped inside the cell, thus enabling measurement of intracellular free radicals (Wang and Joseph, 1999). The addition of hydrogen peroxide to SHSY treated with DCFDA resulted in a time-dependent increase in the fluorescence signal (figure not shown). This induction of fluorescence was inhibited by argpyrimidine in a concentration-dependent manner (Fig. 7), and the fluorescence was completely abrogated by argpyrimidine at a concentration of 0.1 mM.

3.7. Argpyrimidine scavenges superoxide anion

Argpyrimidine was first investigated for its direct effect on xanthine oxidase by measuring its effect on the amount of uric acid generated by the action of xanthine and xanthine oxidase. The amount of uric acid that was produced in the absence and presence of argpyrimidine was 480±30 and 492±46 nmol respectively, suggesting that argpyrimidine does not directly inhibit the xanthine oxidase (data not shown). Argpyrimidine was capable of scavenging superoxide anion formed by the reaction of xanthine and xanthine oxidase in a concentration-dependent manner, and almost complete scavenging was observed at a concentration of 1 mM argpyrimidine (Fig. 8).
Control experiments in the absence of xanthine–xanthine oxidase showed that argpyrimidine (0.1–1 mM) does not directly reduce NBT (Data not shown).

4. Discussion

The present study demonstrated that argpyrimidine, the major end-product of methylglyoxal-induced protein glycation, possesses strong antioxidant properties as evidenced by its ability to inhibit in-vitro lipid peroxidation (induced by hydroxyl radicals, metal ions, and autoxidation), attenuate free-radical-induced DNA-cleavage, and scavenging free radicals such as DPPH, hydrogen peroxide and superoxide anion. In addition, argpyrimidine was also capable of reducing ferric ions to ferrous ions at physiological pH. The ability of argpyrimidine to inhibit the induction of lipid peroxidation at a very early time suggests that argpyrimidine may be functioning as a chain-breaking antioxidant in a manner similar to alpha-tocopherol and butylated-hydroxytoluene.

Increased levels of argpyrimidine have been observed in several pathophysiological conditions such as diabetic nephropathy and neurodegenerative diseases (Thornalley, 1999). Argpyrimidine has been shown to co-localize with end products of lipid peroxidation in spinal cords of patients with neurodegenerative diseases (Shibata et al., 2001). However, it remains unclear whether this increase in argpyrimidine levels is a cause or consequence of the disease conditions. It is possible that argpyrimidine may represent a natural antioxidant defense upregulated by the body to counter these pathophysiological events. However, further extensive studies under in-vivo conditions are required to ascertain such possibilities.

The concentrations of argpyrimidine used here far exceed the physiological concentrations of argpyrimidine present even under pathophysiological conditions and constitute a short-coming of this study. However, the extent of free radicals generated in the models used in these studies is also supra-physiological in nature albeit its low physiological levels and the localization of argpyrimidine at sites of injury may allow it to trap oxygen radicals that are generated under in-vivo conditions. The IC-50 (concentration for 50% inhibition) of 0.73 mM and 0.012 mM (derived from Figs. 3B and 7) for inhibition of lipid peroxidation and scavenging of intracellular-hydrogen peroxide respectively is suggestive of the fact that a lower concentration of argpyrimidine may be sufficient when it is localized intracellularly at the site where free radicals are generated. However, the IC-50 values for inhibition of lipid peroxidation for α-tocopherol was 0.41 mM (data not shown), suggesting that α-tocopherol is more potent than argpyrimidine in inhibiting lipid peroxidation. This difference may be attributed to the higher lipid partitioning of α-tocopherol.

In summary, the present study shows that argpyrimidine has potent antioxidant properties in vitro. Given the fact that argpyrimidine accumulates in sites of injury (eye lens, vascular beds, and neurofilbrillary tangles), it is tempting to speculate that argpyrimidine may represent one of the several biological defenses against oxidative damage in vivo. If this is indeed the case, it would be interesting to develop antioxidants using argpyrimidine as a prototype.

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