Prevention of Oxidative Damage to Lens by Caffeine

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Abstract

Purpose: The primary objective of this study was to examine the possibility of inhibiting oxidative damage to the lens *in vitro* by caffeine.

Methods: Oxidative damage was inflicted by incubating mouse lenses in Tyrode medium containing 0.1 mM Fe_8Br_8 , an iron complex soluble in aqueous medium. Parallel incubations were conducted in the presence of caffeine (5 mM).

Results: Lenses incubated in the medium containing Fe_8Br_8 undergo oxidative stress, as evidenced by the inhibition of $\text{Na}^+ - \text{K}^+$ ATPase-driven rubidium transport and the loss of tissue glutathione and ATP. These effects were prevented in presence of caffeine. That the effects are due to the oxyradicals produced was ascertained further by parallel studies with Tempol (5 mM), a well-known scavenger of reactive oxygen species (ROS) with its activity being more pronounced with hydroxyl radicals as compared to other ROS.

Conclusions: Caffeine was found to be effective in preventing oxidative stress to the lens induced by iron under ambient conditions. The protective effect is attributable to its ability to scavenge ROS, particularly the hydroxyl radical.

Introduction

THE HYPOTHESIS THAT AN intraocular generation of oxygen-free radicals constitutes a significant initiating factor in the genesis of age-associated cataract development is now widely recognized. The proposal was initially based on the findings demonstrating the presence of superoxide dismutase (SOD) in the ocular lens,^{1,2} suggesting that the lens and its surrounding fluids must be generating superoxide, the monovalently reduced species of reactive oxygen, by several autooxidative as well as enzymatic reactions.^{3,4} In addition, it is also generated by electron leakage from the reduced cytochromes and its capture by oxygen during oxidative phosphorylation. Once formed, this oxygen centered free radical dismutates to peroxide—a nonradical form of reactive oxygen species (ROS). This is followed by the interaction of these 2 species generating hydroxyl radical, a reaction catalyzed by redox active metals such as iron and copper.^{5,6} In the case of intraocular tissues and fluids including the lens and aqueous humor, reactive species of oxygen including superoxide and singlet oxygen can also be generated by several photodynamic reactions, explaining the higher incidence of cataracts in areas of the world with higher intensity of solar radiation.⁷ Light and oxygen hence

have been suggested to act synergistically in the onset and progression of age-associated cataract formation.^{8,9} Support for these hypotheses have initially been obtained through lens culture studies in media generating ROS photochemically as well as by nonphotochemical reactions.^{10–13} Such experiments convincingly demonstrate the susceptibility of the lens to ROS in physiological as well as morphological terms. The physiological damage is reflected by an inhibition of its $\text{Na}^+ - \text{K}^+$ ATPase-dependent cation transport activity and loss of glutathione (GSH). It is also reflected by protein oxidation and consequent generation of heavy molecular weight species.¹⁴ Morphologically, the damage is apparent by the loss of its transparency and development of structural abnormalities detected histologically. The end result is cell death by apoptosis.¹⁵ Several nutritional scavengers of ROS such as ascorbate,¹⁶ tocopherols,^{17,18} and bioflavonoids¹⁹ have been shown to protect the lens against such damage. There is some evidence that they have an attenuating effect against formation of cataracts in humans also. However, they have not been shown to be convincingly effective pharmacologically. This could be due to their instability and becoming even pro-oxidant in presence of certain trace metals. In addition, the relative lack of effectiveness can also be attributed

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to their high susceptibility to photodegradation. With this in view, it is highly desirable to develop ROS scavengers that are more stable and physiologically compatible. In a previous study, we have shown that caffeine could effectively protect the lens from damage inflicted by UV.²⁰ We therefore hypothesize that it could be effective in protecting the lens against damage caused by ROS generated by nonphotochemical reactions also as expected under ambient conditions. Since the eventual damage by ROS is inflicted due to the hydroxyl radical generated by metal-catalyzed reactions, emphasis in this study has been given on the effectiveness of caffeine against iron-induced oxidative damage, the reaction conditions being such that the major oxidant produced is OH $\cdot$, with very minor and transient existence of superoxide and hydrogen peroxide.

Methods

All chemicals were procured from Sigma Chemical Company (St. Louis, MO). Medium 199 (Cat # 11043023) was obtained from Invitrogen (Carlsbad, CA). Aqueous soluble iron complex (Fe $_8$ Br $_8$) was kindly supplied by Dr. Naresh Dalal of Florida State University. Lenses were obtained from CD-1 mice. The weight of the animals varied between 25 and 30 g with the lens weight varying between 7 and 9 mg. The research followed the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animal handling procedures were approved by the Institutional Animal Care and Use Committee.

Freshly isolated individual mice lenses were incubated in 1 mL of medium 199 containing $^{86}\text{RbCl}$ as a tracer. The incubation was conducted in a humidified water-jacketed incubator maintained at 37°C and gassed with 5% carbon dioxide in air. The period of incubation was 18 h. Incubations were done in groups labeled as basal, control, and experimental. The basal control group represents lenses incubated in the above medium. Controls represent lenses incubated in the above medium but with the addition of the soluble iron complex Fe $_8$ Br $_8$ (0.1 mM). The experimental groups represent lenses incubated in medium containing the iron complex plus caffeine or Tempol (1-oxyl-2,2,6,6-tetramethyl-4-hydroxypiperidine, Sigma Chemical Co., Cat # 176141) at the concentration of 5 mM. Subsequent to incubation, the individual lenses were gently removed from the medium, examined for any opacity development, rinsed with 100 μL of physiological saline to get rid of any adherent radioactivity, and transferred to tubes for $^{86}\text{Rb}^+$ counting in a gamma counter. An equal amount of the medium was also counted. Subsequent to counting the lenses were homogenized in tubes containing 0.5 mL of ice cold dH $_2$ O, the tubes remaining surrounded by ice. The homogenate was then centrifuged in cold to facilitate the precipitation of the insoluble proteins. The supernatant was then used for determination of ATP and GSH as follows.

ATP determination

This was determined luminometrically.²¹ An aliquot (~50 μL) of the aqueous lens extract prepared as above was mixed with 100 μL of luciferin–luciferase mixture (Sigma Chemical Co., Cat # FLE 250) and the luminescence read at the maximum peak. ATP standards were run simultaneously.

GSH determination

One hundred percentage of trichloroacetic acid (TCA) was added to the tube containing the neutral extract to a concentration of 5%.²² The acid soluble extract thus obtained was then aspirated for determination of GSH using Ellman's reaction as follows. One hundred microliters of the TCA extract was added to tubes containing 300 μL of 0.6 M disodium hydrogen phosphate. This was followed by the addition of 100 μL of the Ellman's reagent prepared by dissolving 4 mg 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in 1% trisodium citrate solution. The yellow color produced was measured spectrophotometrically at 412 nm after allowing about 10 min for the reaction to get completed.

Results

The lens being avascular derives most of its nutritional and metabolic requirements through membrane transport. Its homeostasis hence is highly dependent on the integrity of its cell membranes. Many previous studies have demonstrated that one of the early hallmarks of cataract formation is indeed damage to its cell membranes. This has also been found to be true in experimental cataracts such as those initiated by diabetes and galactosemia.²³ It is also one of the earliest manifestations of cataracts induced by exposure of the lens to ROS, suggesting that membrane oxidation is an early event in the induction of cataracts initiated by oxidative stress also. The initial damage appears to be due to the loss of membrane sulfhydryls and structural aberrations caused by lipid peroxidation.^{24,25} Both these processes have an adverse effect on the ability of the tissue to accumulate K $^+$ against the concentration gradient with concurrent extrusion of Na $^+$, maintaining high intracellular K $^+$ and low Na $^+$. With this in view, the lens damage caused by incorporation of the iron complex in the medium of incubation was initially assessed by measurement of the uptake of $^{86}\text{Rb}^+$. Since the transport of this ion (acting as a surrogate of the K $^+$) occurs against its concentration gradient, the effect of iron toxicity was measured in terms of the ratio of its concentration (CL/CM) attained between the lens water (CL) and the medium of incubation (CM).

As summarized in Figure 1, the basal ratio, attained in the lenses incubated without the iron complex was close to 23, matching the ratio of the K $^+$ concentration between the cellular and extracellular compartments in general. In the presence of the iron complex this ratio was substantially depressed (~7), suggesting an inhibition of the Na $^+$ –K $^+$ pump because of oxidative stress. Addition of caffeine to the medium was highly attenuative, the ratio in this case was significantly higher (~14) than in its absence (~7). That the inhibitory effect of the iron complex was due to the ROS generated in the medium, particularly the hydroxyl radical, was apparent from studies with Tempol, an established scavenger of ROS. As summarized in Figure 2, its addition to the medium was also highly protective. The distribution ratio of the ion in this case was equal to the basal control values, the iron effect being almost completely annulled, proving that the toxicity of the iron was due to ROS generation. It was interesting to note that the level of hydrogen peroxide in the medium incubated with the iron complex alone was hardly detectable, being present only in traces if at all, strongly suggesting that the iron complex rapidly converts the peroxide

FIG. 1. Inhibition of active transport of $^{86}\text{Rb}^+$ by Fe_8Br_8 complex. Prevention by caffeine (5 mM). Mice lenses were incubated in Tyrode medium as described in Methods. The data are expressed as mean ratio of concentration (CL/CM) attained between the lens water (CL) and the medium of incubation (CM) $\pm$ S.D.; $n \geq 4$; $P < 0.001$ between control group and the group incubated with Fe_8Br_8 *, as well as that between groups incubated with Fe_8Br_8 in the absence and presence** of caffeine.

that might have been initially produced by O_2^- dismutation, to hydroxyl radical.

Further evidence of the induction of oxidative stress to the lens by the iron complex and the preventive effect of caffeine was apparent from measurements of GSH, an

FIG. 2. Preventive effect of Tempol (5 mM) against the iron complex (Fe_8Br_8) induced damage to lens. The results are expressed as ratio of concentration (CL/CM) attained between the lens water (CL) and the medium of incubation (CM) as affected by the iron complex in the absence and presence of Tempol. Controls were run without the iron complex. Addition of Tempol was preventive. P values between control group and the group incubated with the iron complex*, as well as that between groups incubated with Fe_8Br_8 in the presence** and absence of Tempol were all < 0.001 ; $n \geq 4$.

important antioxidant reserve. As summarized in Figure 3, the level of this $-\text{SH}$ tripeptide was substantially lowered on incubation with the iron complex. Caffeine substantially prevented such lowering induced by oxidation, the oxidized product (GSSG) leaving the lens rapidly as has been shown previously.²⁶

The inhibition of rubidium uptake could hence involve several factors such as the oxidative changes in the components of the membrane such as the $\text{Na}^+ - \text{K}^+$ ATPase as well as loss of ATP required for the activity of this transporter. It is believed that the immediate effect of ROS generated in the medium is on the enzyme. This is followed by several metabolic aberrations and intracellular changes such as oxidation of structural and enzymatic proteins with the end result of protein aggregation and metabolic inhibition. Caffeine has been found to inhibit the latter processes as evident by measurement of the levels of tissue ATP. As summarized in Figure 4, its level underwent substantial lowering in the presence of the iron complex, the level reaching about one-fourth of the controls. In the presence of caffeine, the level remained well maintained as was the case with the maintenance of the levels of GSH and the rubidium transport activity. Addition of Tempol to the medium also had similar effects (Figs. 5 and 6).

Discussion

Several previous laboratory and epidemiological studies have implicated oxidative stress as one of the major factors in the genesis of senile cataract. One of the objectives of several lens studies is hence to examine the feasibility of preventing its formation pharmacologically by use of nutritional and metabolic antioxidants. The latter can sometimes be of additional benefit due to their action as metabolic agonist. Progress in such pharmacological studies, however, has so far been limited. While several studies suggest the feasibility of attenuating cataract formation by nutritional antioxidants, their effectiveness appears to be limited because of their susceptibility to photochemical degradation in the eye. The primary objective of this study was hence to

FIG. 3. Preventive effect of caffeine against depletion of glutathione (GSH). The level was substantially lowered in the presence of the iron complex*. Addition of caffeine (5 mM) was highly preventive**. $P < 0.001$; $n \geq 4$.

FIG. 4. Maintenance of ATPase-driven rubidium transport levels by caffeine (5 mM). Incubation with iron but without caffeine depleted the tissue of its ATP content significantly*. Addition of caffeine was highly preventive**. $P < 0.001$; $n \geq 4$.

determine the efficacy of caffeine, a much more stable compound and known to scavenge ROS, in preventing oxidative damage to the lens. The study was also prompted by the fact that it is a common constituent of many beverages and therefore relatively well tolerated physiologically. In a previous study, we have demonstrated that it can protect the lens against UV-induced development of opacification. Since in most cases, oxidative stress involves transition metal-induced redox cycling generating ROS, the present studies were designed to determine the possible protective effect of caffeine specifically against iron-induced oxidative

damage. The study was greatly facilitated by the availability of an unchelated soluble iron complex (Fe_2Br_9), which is capable of undergoing redox cycling without becoming insoluble at physiological pH of 7.4–7.6 wherein most of the iron salts precipitate out as hydroxide. This compound has not been used so far for studying oxidative stress, at least in the lens. As described in "Results," its addition to the medium was significantly deleterious to the physiology of the tissue indexed by the inhibition of its sodium–potassium pump, inhibition of tissue metabolism indexed by lowering of ATP content, and loss of GSH. That these physiological aberrations are induced by unwanted reactions initiated by oxyradicals were proven by the protective effect of Tempol, which scavenges superoxide (rate constant $6 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$)²⁷ as well as hydroxyl radicals (rate constant $1 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$).²⁸ Caffeine has also been previously reported to scavenge both these ROS, with rate constants being $7.3 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ for reaction with $\text{OH}^\cdot$ and $7.5 \times 10^1 \text{ M}^{-1}\text{s}^{-1}$ for superoxide.^{29,30} Hence, as expected, addition of caffeine to the medium of incubation offered significant protection against oxidative damage to the cation transporter as well as to the loss of GSH caused by incubation of the tissue with the iron complex. The levels of ATP were also better maintained. The maintenance of their levels GSH by caffeine even in the presence of Fe complex strongly rules out the possibility that caffeine could act as a pro-oxidant in this tissue.

Since the rate constant of the reaction of caffeine with superoxide is lower than the spontaneous dismutation rate of superoxide (rate constant $\sim 10^7$), the effect of caffeine appears primarily to be due its reaction with $\text{OH}^\cdot$. This is also apparent from only traces of hydrogen peroxide being detected—the reaction proceeding fast to generate $\text{OH}^\cdot$. The product formed by the reaction of caffeine with $\text{OH}^\cdot$.

FIG. 5. Maintenance of ATPase-driven rubidium transport by Tempol (5 mM). The depletion of ATP by iron complex was prevented substantially by Tempol. The P value between the group incubated with iron alone* and iron + Tempol** was < 0.001 , $n \geq 4$ in each group.

FIG. 6. Inhibition of glutathione (GSH) loss by Tempol (5 mM). The content of this tripeptide is significantly decreased by incubation with the iron complex* as compared to the basal controls. Addition of Tempol** was highly preventive, P value being < 0.001 ; $n \geq 4$.

is trimethyl urate, also known to scavenge ROS.³¹ Caffeine would therefore protect the tissue by its own ability to scavenge OH[•] radical as well as by generating a product that can offer an additional protective effect.

In summary, therefore, caffeine has been found effective in protecting the lens against damage induced by ROS generated by redox cycling of trace metals such as iron. While this xanthinoid has several other known biochemical effects on tissues, such as its ability to maintain levels of c-AMP by inhibiting phosphodiesterase and modulating cellular levels of calcium, the present studies seem to suggest that the protective effect is primarily related to its ability to scavenge the ROS, particularly the hydroxyl radicals.

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