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Molecular identification and cellular localization of a potential transport system involved in cystine/cysteine uptake in human lenses

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ABSTRACT

In this study we have sought to identify whether cystine uptake mechanisms previously identified in the rat lens are also found in the human lens. Using a combination of reverse transcriptase PCR, Western blotting and immunohistochemistry, we show that the light chain subunit of the cystine/glutamate exchanger (X_C-), xCT, and members of the glutamate transporter family (X_{AG}) which include the Excitatory Amino Acid Transporter 4 (EAAT4) and the Alanine Serine Cysteine Transporter 2 (ASCT2) are all present at the transcript and protein level in human lenses. We demonstrate that in young lenses xCT, EAAT4 and ASCT2 are expressed in all regions indicating that a potential cystine uptake pathway similar to that found in the rat might also exist in human lenses. However, with increasing age, the immunolabeling for all transporters decreases, with no xCT labelling detected in the centre of old donor lenses. Our results show that X_C- and EAAT4/ASCT2 may work together to mediate cystine uptake in the lens core of young human lenses. This suggests that the lens contains uptake mechanisms that are capable of accumulating cystine/cysteine in the lens centre where cysteine can be used as an antioxidant or cystine utilised as a source for protein-S-S-cysteine (PSSC) formation to buffer against oxidative stress. With increasing age, transporters in the lens core undergo age dependent post translational modifications. However, despite processing of these transporters with age, our results indicate that this cystine uptake pathway could account for the increased PSSC levels previously observed in the nucleus of older human lenses.

1. Introduction

The most common form of cataract is age related nuclear (ARN) cataract and its incidence is expected to only increase as the population ages. A key feature of ARN cataract is protein oxidation characterised by a loss of protein sulfhydryl groups and extensive oxidation of methionine and cysteine residues (Truscott, 2005). An early indicator of oxidative stress is the increased formation of protein thiol mixed disulfides generated by thiol/disulfide exchange between protein thiols (PSH) and oxidised glutathione (GSSG) or oxidised cysteine (CSSC) resulting in the formation of protein-S-S-glutathione (PSSG) and protein-S-S-cysteine (PSSC), respectively (Lou, 2003). Traditionally, these mixed disulfides were thought to precede a cascade of events that include protein disulfide cross linking, loss of protein solubility, high molecular weight protein aggregation and ultimately cataract formation (Lou, 2003).

Although PSSG and PSSC are considered potential precursors for cataract formation, they are also present in normal clear lenses (Lou, 1997) where they are believed to act in maintaining protein redox regulation by protecting exposed SH groups from irreversible oxidation. PSSG and PSSC can be dethiolated by repair enzyme systems such as thioltransferase to restore original protein or enzyme structure and function (Lou, 2000; Neal et al., 1998; Willis and Schleich, 1996). However, unfortunately with advancing age or exposure to prolonged oxidation there is a profound increase in PSSG and PSSC formation (Lou, 2003; Lou and Dickerson, 1992; Takemoto, 1996) which when coupled to reduced repair enzyme function (Xing and Lou, 2010) leads to protein conformational changes that favour the formation of protein–protein cross links (PSSP) that result in protein aggregation and cataract formation.

The relative amounts and regional distributions of PSSG to PSSC vary between species (Lou, 2000). In the rat lens, the majority of PSSG is located in the cortical region, while most of the PSSC is

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located in the nucleus. In contrast, in human lenses, both PSSG and PSSC are preferentially found in the nucleus (Lou, 2000). The source for PSSG is most likely derived from GSH which is typically found at mM concentrations in both rat and human lenses (Lou, 2003). In both species, lens GSH levels are highest in the outer cortex, before gradually decreasing towards the lens centre. In contrast, the concentration gradient for cysteine is opposite to that of GSH, with cysteine levels being highest in the nucleus relative to the cortex (Dickerson et al., 1997; Veltman and Lou, 1993). However, despite this gradient, the levels of free cysteine in the lens are in the nM range for both rat and human (Dickerson et al., 1997; Veltman and Lou, 1993) and at these levels cannot account for the relative high concentration of PSSC observed in the lens nucleus (Lou, 2003). This suggests that the lens contains uptake mechanisms that are capable of accumulating cystine/cysteine in the lens centre which can then be used as a source for PSSC formation to buffer against oxidative stress.

This raises the question of how this cysteine is specifically accumulated in the lens nucleus. Persa et al. (2004) have shown that cysteine levels in the pig lens and in human lens epithelial cells can be controlled via the transsulfuration pathway. The identification of the rate limiting enzyme in the transsulfuration pathway was identified in the nucleus of young pig lenses indicating that this pathway could contribute to the accumulation of cysteine in the lens nucleus (Persa et al., 2004). In the rat lens, an alternative pathway for accumulating free cystine in the lens centre has been identified that utilises the direct uptake of cystine, the oxidized and more stable form of cysteine, followed by its subsequent reduction to cysteine (Lim et al., 2005).

We have shown in the rat lens that this cystine pathway is mediated by the cystine/glutamate exchanger (X_C^-) and members of the glutamate transporter X_{AG} family (Lim et al., 2005, 2006). X_C^- exchanges extracellular cystine for intracellular glutamate and works in partnership with the X_{AG} family to recycle extracellular glutamate in order to maintain the glutamate concentration gradient (Bannai, 1986; McBean, 2002; Sato et al., 1999). We showed that the light chain subunit of X_C^-, referred to as xCT, is present throughout the rat lens, but its glutamate transporter partner switches from the Excitatory Amino Acid Transporter 4 (EAAT4) in the lens cortex to the Alanine Serine Cysteine Transporter 2 (ASCT2) in the lens nucleus (Lim et al., 2005, 2006). In order to assess transport function, we utilised cystine specific antibodies to map the cellular accumulation of free cystine and correlated this to the membrane expression of xCT in the different regions of the lens (Li et al., 2007). Using this approach, accumulation of cystine and the membranous expression of xCT served as an indirect measure of cystine uptake. We showed that a bimodal pattern of cystine uptake exists in which cystine levels were highest in the outer cortex, decreased to a minimum in the inner cortex before increasing in the core relative to the inner cortex (Li et al., 2007). This bimodal pattern cannot be explained by passive diffusion and indicates that cystine is preferentially delivered to the nucleus via the sutures.

Based on these results, we have proposed that in the cortex, X_C^- and EAAT4 work together to mediate the uptake of cystine, where it is rapidly reduced to cysteine and then utilised for GSH/protein synthesis (Lim et al., 2005). On the other hand, in the lens core, which is not capable of GSH synthesis, we have suggested that X_C^- and ASCT2 work together to mediate the accumulation of cystine (Lim et al., 2006) where if it is subsequently reduced to cysteine it can act as an antioxidant or alternatively, the accumulated cystine can serve as a source for PSSC formation The switch in glutamate transporter partner from EAAT4 in the cortex to ASCT2 in the core, suggest that xCT partners with different glutamate transporters in order to maximise cystine uptake within different regions of the lens.

Table 1
Primer sets.

Protein	Oligonucleotide	Expected PCR product size
Cx50[a] (GenBank Acc. No. NM_005267	Sense (24 b, position 1000) TCCCGGGGCTACCAAGAGACACTG Antisense (27 b, position 1318) CCTGGCTCGGCTGCTGGCTTTGCTTAG	345 bp
xCT[a] (GenBank Acc. No. AF252872)	Sense (21 b, position 660) CCTGGCATTTGGACGCTACAT Antisense (22 b, position 820) TCAGAATTGCTGTGAGCTTGCA	161 bp
EAAT4[a] (GenBank Acc No. NM_005071)	Sense (19 b, position 31) TGTTCCTTCGGGAGAGCGG Antisense (19 b, position 613) GTTACCACCCTCGTGCTGT	601 bp
ASCT2[a] (GenBank Acc. No. NM_005628)	Sense (18 b, position 1692) CCGCTGATGATGAAGTGC Antisense (18 b, position 2181) CCCCCGATAGTGTTTGAG	507 bp

[a] Primer sets for Cx50[19], xCT[11], EAAT4[20],and ASCT2[21] were taken from the literature.

In this paper, we have extended our previous work on rats to human and have investigated whether human lenses contain transporters to accumulate cysteine in the different regions of the lens.

2. Materials and methods

2.1. Reagents

A carboxyl terminal isoform specific EAAT4 antibody and its corresponding control peptide were purchased from Alpha Diagnostics International (Texas, USA). Amino terminal tail-specific antibodies for xCT and ASCT2 were purchased from CosmoBio (California, USA) and Chemicon International (California, USA), respectively. No control peptide was available for the xCT and ASCT2 antibody. The anti-rabbit cystine antibody was a generous gift from Dr Robert Marc (University of Utah School of Medicine, Utah, USA). The goat anti-rabbit Alexa 488 secondary antibody and the membrane marker wheat germ agglutinin conjugated to tetramethyl rhodamine isothiocyanate (WGA-TRITC) were both obtained from Molecular Probes Inc (Eugene, USA). Phosphate buffered saline (PBS) was prepared from PBS tablets (Sigma Chemical Company, St Louis, USA). Unless otherwise stated all other chemicals were from Sigma.

2.2. Lenses

Human lenses were obtained from donor eyes courtesy of the New Zealand National Eye Bank within 24–48 h of death. On examination, all lenses were transparent throughout, as determined by the clarity of the grid lines behind the lens. Human lens work was conducted in compliance with the Declaration of Helsinki and was approved by the Northern X Regional Ethics Committee (Ref: NTX/07/08/079).

2.3. Reverse transcriptase polymerase chain reaction

The lens epithelium and the outer cortex were carefully removed and total RNA isolated using Trizol (Gibco, New York, USA) and a Pure-Link® RNA mini kit (Invitrogen, California, USA) yielding ~300 ng/μl of fibre total RNA from each lens which was quantified using a NanoDrop device (ND-1000; Thermo Scientific). Approximately 2 μg of total RNA was reverse-transcribed using SuperScript III First-Strand Synthesis SuperMix (Invitrogen). A control reaction