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Cystine/glutamate transporter, system x_c^-, is involved in nitric oxide production in mouse peritoneal macrophages

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ABSTRACT

The amino acid transport system x_c^- is important for maintaining intracellular glutathione levels and extracellular redox balance. The main component of system x_c^-, xCT, is strongly induced by various stimuli, including oxidative stress and bacterial lipopolysaccharides (LPS) in macrophages. In the present study, we investigated the production of nitric oxide by LPS-stimulated mouse peritoneal macrophages isolated from both xCT-deficient and wild-type mice. After culturing macrophages in the presence of LPS for 24–48 h, nitrite levels in the medium of xCT-deficient macrophages were significantly decreased compared to that of wild-type cells. However, the transport activity of arginine, a precursor of nitric oxide, and the expression of nitric oxide synthase 2 in xCT-deficient macrophages were similar to those of wild-type cells. When wild-type macrophages were cultured in the medium that contained no cystine, nitric oxide production was decreased to the level similar to that of the xCT-deficient macrophages. When xCT-deficient macrophages were cultured with 2-mercaptoethanol, intracellular cysteine levels were increased and nitrite accumulation in the medium was significantly increased. On the other hand, when these cells were cultured with buthionine sulfoximine, an inhibitor of glutathione synthesis, nitrite accumulation in the medium was essentially unchanged, although intracellular glutathione levels were very low. Reactive oxygen species levels in xCT-deficient macrophages were higher than those of wild-type cells, and treatment with LPS caused an increase in oxidative stress in both cells. These results suggest that intracellular cysteine supplied by xCT contributes to nitric oxide production and the reduction of oxidative stress in macrophages.

1. Introduction

Macrophages produce large amounts of reactive oxygen species (ROS) and nitric oxide (NO) in response to inflammatory stimuli such as bacterial lipopolysaccharides (LPS) and interferon γ, and function to protect the host from bacterial infection [1,2]. In macrophages, NO synthase 2 (NOS2) is induced by various stimuli, including LPS and interferon γ, which cause the production of large amounts of NO. Recent studies have revealed that NO produced by macrophages functions, not only as an antimicrobial, tumoricidal, and tissue-damaging effector molecule operating in the innate immune system, but also as an effector of adaptive immune responses and cyto-protection [3]. Although NO itself plays multiple physiological roles, it also reacts with other gaseous molecules and is converted to reactive nitrogen oxide species (RNOS). Among the known RNOS, peroxynitrite ($ONOO^-$), which is formed by the reaction of NO with superoxide, exerts oxidative damage to many biological molecules [4]. Acting in coordination, antioxidative and redox molecules protect cells against oxidative and nitrosative stress by eliminating ROS and RNOS.

Glutathione is the most abundant non-protein thiol that is produced in cells and plays pleiotropic roles, such as antioxidation and the detoxification of toxicants. The antioxidative functions of glutathione are effectively expressed by donating electrons to peroxides via glutathione peroxidase (GPX) [5]. Thus, an insufficiency of glutathione triggers the redox imbalance and makes cells more vulnerable to oxidative insults, leading to cell death.

Intracellular glutathione levels are maintained by *de novo* synthesis from constituent amino acids, glutamate, glycine, and cysteine that is catalyzed by the action of γ-glutamylcysteine synthetase (γGCS) and glutathione synthetase (GSS) as well as by recycling oxidized

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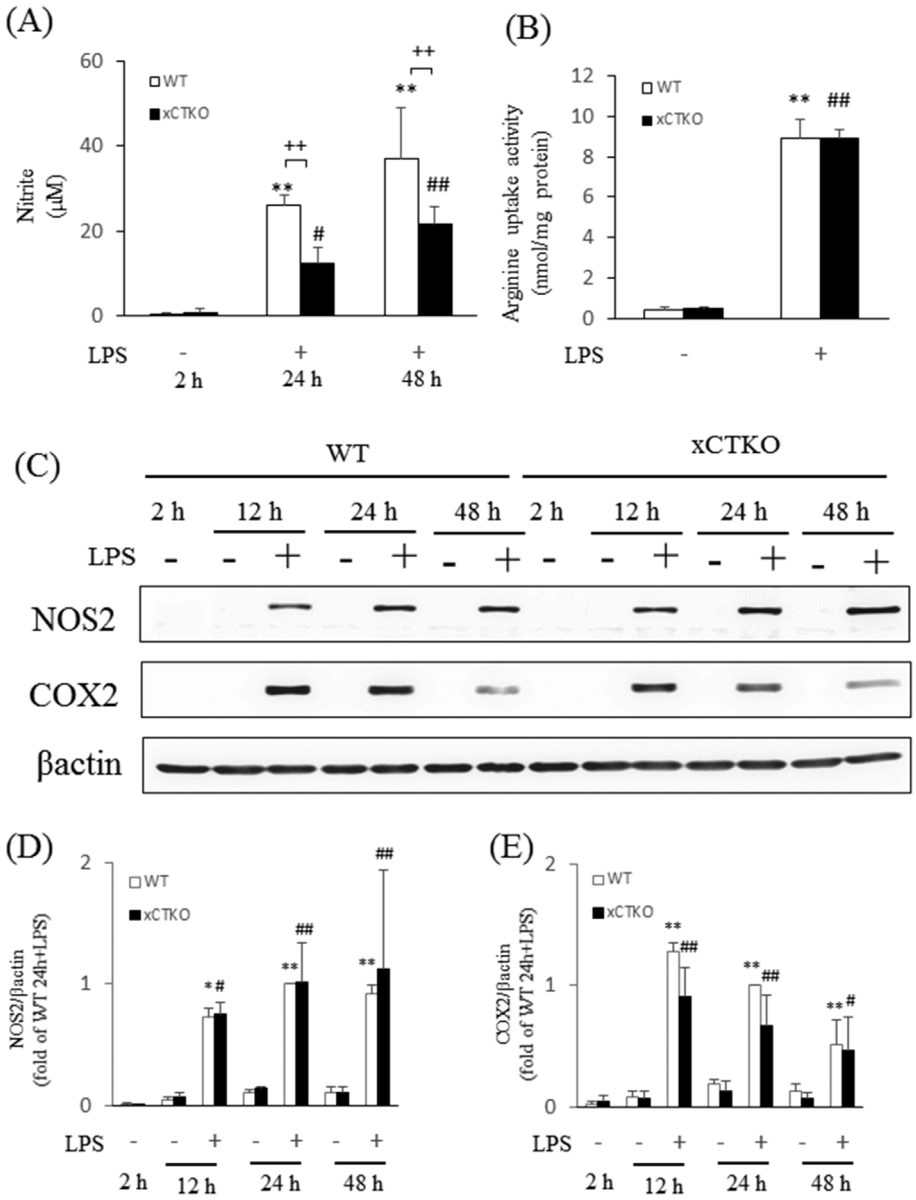

Fig. 1. NO production by xCT-deficient macrophages. (A) Nitrite in the culture medium was assayed by the Griess method. Columns and bars represent the mean ± SD (n = 7–10). ($^{**}P < 0.01$, compared with WT at 2 h; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with KO at 2 h; $^{++}P < 0.01$). (B) [^{14}C]Arginine was used to assay the rate of arginine uptake by macrophages after a 24 h culture period with or without LPS. Columns and bars represent the mean ± SD (n = 7–10). ($^{**}P < 0.01$, compared with WT without LPS; $^{\#\#}P < 0.01$, compared with KO without LPS). (C) Immunoblot analyses were performed using anti-NOS2 or anti-COX2 antibodies as well as β-actin. Quantification was performed on each protein band and normalized to the corresponding β-actin band; NOS2 (D) and COX2 (E). Columns and bars represent the mean ± SD (n = 3). ($^{*}P < 0.05$, $^{**}P < 0.01$, compared with WT at 2 h; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with KO at 2 h).

glutathione by glutathione reductase (GSR) in a NADPH-dependent manner [6]. A redox-sensitive transcriptional regulatory factor, Nrf2, regulates the expression of the enzymes that are involved in both the *de novo* synthesis and reductive recycling of the oxidized glutathione [7,8]. Among three substrate amino acids, cysteine is generally maintained at low levels in many types of cells and thus constitutes a rate-limiting factor for the synthesis of glutathione [9].

Several systems, including amino acid transporters and metabolic protein degradation, function to supply cysteine to cells [10]. In some types of cells, cysteine can be supplied via the transsulfuration pathway in association with methionine metabolism [11]. A reduced form of extracellular cysteine is transported into cells via neutral amino acid transporters, e.g., ASCTs and LAT2 [12,13]. The oxidized form of cysteine, i.e., cystine, is taken up by cells via system x_c^-, which is composed of two protein components; xCT (SLC7A11) and 4F2hc (SLC3A2) [14]. In many cultured cells, xCT is induced by various stimuli such as electrophilic agents [15], amino acid deprivation [16], bacterial lipopolysaccharide [17], and contributes to the maintenance of intracellular glutathione level. xCT is the main component of the system x_c^- and mediates the transport of cystine and cystathionine into cells in exchange with glutamate [14,18].

xCT is constitutively expressed in a limited number of organs, such as the thymus, spleen, and cerebral meninges [19]. Upon oxidative stress, xCT is induced in alveolar macrophages, the kidney, and liver, and hence a xCT deficiency aggravates the oxidative damage in these organs [20–23]. xCT-deficient (KO) mice show healthy phenotypes except for a redox imbalance in the blood plasma [24]. Due to the oxidative conversion of cysteine to cystine in the culture medium, intracellular glutathione levels depend on the extent of cystine uptake via xCT in many types of cultured cells under routine culture conditions. Mouse embryonic fibroblasts (MEF) isolated from KO mice are unable to survive under routine culture conditions due to decreased glutathione levels [18,24].

We previously reported that the activities of system x_c^- and system y^+, which mediates the transport of cationic amino acids, are strongly induced by a very small amount of LPS in macrophages [17,25]. Because arginine is a substrate of system y^+ and a precursor of NO, it seems rational that system y^+ and NOS2 are simultaneously induced by LPS. The question arises as to whether the simultaneous induction of system x_c^- and system y^+ has any physiological and/or pathophysiological significance. In the present study, we compared NO production by peritoneal macrophages isolated from both KO and wild-type (WT)