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Dipotassium glycyrrhizate via HMGB1 or AMPK signaling suppresses oxidative stress during intestinal inflammation

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ABSTRACT

Aims: Oxidative stress and inflammation are always associated. Appropriate management of oxidative mediators may represent a therapeutic strategy to reduce inflammation, and use of antioxidant can be protective against inflammatory diseases. Glycyrrhizin (GL) plays an anti-inflammatory and antioxidant effect by inhibiting high mobility group box 1 (HMGB1) or 11-β-hydroxysteroid dehydrogenase type II (11βHSD2) enzyme. In this study, the potential role of dipotassium glycyrrhizate (DPG), a salt of GL, to reduce oxidative stress in intestinal inflammatory condition was investigated *in vivo* and the mechanism of action of DPG was studied *in vitro*.
Results: In a colitis mouse model DPG affected oxidative stress reducing iNOS and COX-2 expression, as well as NO and PGE2 levels. By means of LPS-stimulated macrophages we found that DPG inhibited the expression of pro-inflammatory cytokines and reduced iNOS and COX-2 expression in a time dependent manner, through two different ways of signal. DPG reduced, at a later time, both iNOS and COX-2, through a mechanism HMGB1-dependent, and at an earlier time only COX-2, through a mechanism AMP-activated kinase (AMPK)-phosphorylation-mediated.
Conclusion: DPG has a protective effect on colitis and inflammation through the inhibition of oxidative stress. This study clarifies the two-ways mechanism by which DPG inhibits iNOS and COX-2 during inflammation and demonstrates for the first time that AMPK is a target of DPG. Uncovering this mechanism is significant to clarify the relationship between energy homeostasis and anti-oxidative responses and suggests that DPG could play a relevant role in the development of new therapy against inflammatory diseases associated to oxidative stress.

1. Introduction

The oxidative stress has been implicated in the pathogenesis of disorders associated with diverse human inflammatory diseases [1]. Important players of the oxidative stress process are the enzymes cyclooxygenase type 2 (COX2) and inducible nitric oxide synthase (iNOS), whose expression is directly linked to the generation of high levels of their pro-inflammatory mediators, prostaglandin E2 (PGE2) and nitric oxide (NO), respectively. PGE2 is generally recognized as a mediator of active inflammation, promoting local vasodilatation, attracting and activating neutrophils, macrophages, and mast cells at early stages of inflammatory response [2]. NO has cytotoxic properties aimed against pathogenic microbes, alongside potentially damaging effects on host tissues as interacts with molecular oxygen and superoxide anion to produce toxic reactive nitrogen species [3]. Appropriate management or inhibition of these mediators may represent a therapeutic strategy to reduce inflammation, and use of antioxidant can be protective in the setting of experimental inflammation.

The glycoconjugated triterpene glycyrrhizin (GL), a major active constituent of *Glycyrrhiza glabra* root, is a compound that has been associated with numerous pharmacological effects, including anti-inflammatory, anti-viral, anti-tumor, and hepatoprotective activities, and is commonly used in Asia to treat patients with chronic hepatitis [4–7]. Literature data report that GL inhibits 11-

Abbreviations: HMGB1, high mobility group box 1; 11βHSD2, 11-β-hydroxysteroid dehydrogenase type II; DPG, dipotassium glycyrrhizate; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; PGE2, prostaglandin E2; NO, nitric oxide; IBD, inflammatory bowel disease; AMPK, AMP-activated protein kinase; DSS, dextran sodium sulphate; C-C, Compound-C.

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β-hydroxysteroid dehydrogenase type II (11βHSD2) activity, causing cortisol accumulation [8]. Cortisol, in turn, binds glucocorticoids receptors inhibiting COX-2 expression [8]. It was also reported [9] that GL binds directly to HMGB1 (Kd ~ 150 μM), inhibiting its extracellular pro-inflammatory activity.

High-mobility group box 1 (HMGB1) is a highly conserved inflammatory cytokine-like alarmin that is variably expressed in many cell types [10]. The 25 kDa protein was originally discovered as a nuclear protein, although in inflammatory conditions, HMGB1 is actively secreted from immune cells in the extracellular matrix, where it behaves as a pro-inflammatory cytokine.

Therefore, HMGB1 functions change in relation to its location, as well as to its post-translational modifications [11]. HMGB1 is typically localized in the nucleus; however, when post-translational modifications occur, like acetylation of lysine-residues, it is translocated to the cytoplasm and released from the cell [12].

HMGB1 is considered as a late mediator of inflammation [13]; the protein is released in a time-dependent fashion in culture following LPS treatment. A significant increase of the secreted HMGB1 has been observed in the culture medium after 12–24 h of LPS exposure [14].

Outside the cell, HMGB1 acts as a pro-inflammatory responder to exogenous factors (*e.g.*, infection and stress). HMGB1 is actively released from various cells in response to oxidative stress, bacterial antigens, cytokines, or tissue injury [15–16] and, passively, by necrotic cells [17].

HMGB1 is actually considered a potent inflammatory mediator and implicated in several inflammatory and auto-immune disorders, such as sepsis, rheumatoid arthritis, lupus erythematosus, myositis, diabetes, and ultimately, inflammatory bowel disease (IBD) [18–23]. Recent studies showed that HMGB1 plays important roles also in oxidative stress-associated diseases [24]. Moreover, antioxidants such as ethyl pyruvate [25], quercetin [26], and green tea [27] are protective in the setting of experimental inflammation, partially through attenuating systemic HMGB1 accumulation.

We previously showed that dipotassium glycyrrhizate (DPG), a salt of GL, significantly reduces the DSS induced colitis in mice, without adverse side effects [28]. Our results showed that administration of DPG causes a remarkable reduction of acute colitis, both at macroscopic level (decreased body weight loss and large intestine length, recovery of clinical and histological scores) and in terms of reduction in inflammation (down-regulation of TNF-α, IL-1β and IL-6). These effects are partially mediated by HMGB1 inhibition [28].

Since there is a known relationship between HMGB1 and inflammation as well as between the latter and oxidative stress, the aims of the present study are: to investigate *in vivo* the potential of DPG to reduce oxidative stress in inflammatory condition as well as to study *in vitro* the mechanism of action of DPG. For this purpose expression levels of iNOS and COX-2, and of their mediators, involved in oxidative stress, NO and PGE2, were analyzed *in vivo*, in a mouse model of colitis and *in vitro* by utilizing LPS-stimulated macrophages. Our data show that DPG, during inflammation, affects oxidative stress reducing iNOS and COX-2 expression, as well as NO and PGE2 levels. We found that DPG reduces, at a later times, both iNOS and COX-2, through a mechanism HMGB1-dependent; and at earlier times only COX-2 through a mechanism AMPK-phosphorylation-mediated. In this regard, we identify for the first time AMP-activated protein kinase (AMPK) as a new target of DPG, which is able to activate the enzyme after LPS treatment. According to the literature data, activation of AMPK by phosphorylation inhibits COX-2 expression [29].

Due to the apparent lack of adverse side-effects of DPG, and to its effect on inflammation and oxidative stress, we believe that these findings may open new perspectives on the development of alternative therapies for inflammatory mediated disease.

2. Materials and methods

2.1. Ethic statement

Experimental procedures were previously approved by the Italian Ministry of Health and the study was carried out in accordance to the Italian regulations on animal welfare. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Italian National Agency for New Technology, Energy and Sustainable Economic Development (ENEA) (Permit Number: 131/2012-B).

2.2. Cell culture

The murine macrophage-like cell line, RAW264.7, was cultured in RPMI 1640 medium contained 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (EuroClone S.p.A, Milan, Italy) at 37 °C, 5% CO_2. Cell line was purchased from ATCC (Rockville, MD, USA). Cells were seeded with a cell number of 0.5 × 105 per well in six multiwell plates. Induction of inflammatory response was performed by adding to culture medium (without serum) 1 μg/ml LPS (Sigma, St. Louis, MO) or 1 μg/ml BoxB, the truncated form of the HMGB1 protein consisting of the B box domain, (HMGBiotech, Milan, Italy) in presence or in absence of 300 μM DPG (Sigma) dissolved in PBS (EuroClone). Rabbit anti-murine HMGB1 antibody (1:1000; Sigma) was used to block HMGB1 in cell culture medium and a specific antibody of the same isotype (Rabbit IgG, 1:1000; Sigma) was used as a negative control. To inhibit AMPK-kinase phosphorylation cells were treated with dorsomorphin (compound C, 6-[4-[2-(1-piperidinyl) ethoxy]phenyl]-3-(4-pyridinyl) pyrazolo[1,5-a]pyrimidine; Abcam, Cambridge, United Kingdom) 10 μM at the indicated time points.

Total RNA and proteins were extracted for real-time PCR (RT-PCR) and western blot analyses. Supernatants were collected after 4, 8, 24 and 48 h, briefly centrifuged, and NO, PGE2 and cortisol levels analyzed by colorimetric assay and ELISA assay, respectively.

2.3. Animals

C57BL/6 female mice (8–9 weeks of age; Harlan Laboratories, Udine, Italy) were housed in collective cages at 22/21 °C under a 12-hour light/dark cycle and with food and water provided *ad libitum.* Acute colitis was induced through administration of dextran sodium sulphate (DSS, molecular mass, 36,000–50,000 Da, MP Biomedicals, Santa Ana, CA), dissolved in autoclaved drinking water, for 7 days. Mice, 5 for group, were randomly divided into three experimental groups: the control group received regular drinking water; DSS-treated group was given a solution with 3% (w/v) DSS; a group received a combined treatment with 3% DSS and 8 mg/kg/day DPG (DMG Italia Srl, Pomezia, italy), diluted in PBS, by oral gavage. Mice were daily checked for behavior, body weight, stool blood and consistency. The 7th day, blood samples from submandibular vein were collected from each animal and stored at −80 °C. The same day, animals were euthanized, colons were removed and frozen in liquid nitrogen for further analyses.

2.4. Real-time PCR

Total RNA was isolated from cell line RAW264.7 or from mouse colonic tissues using the RNeasy kit (QiaGen GmbH, Hilden, Germany), and 1 μg of total RNA was reverse transcribed by a IScriptTM cDNA Synthesis Kit (BioRad, Hercules, California, U.S.A). The RT-PCR amplifications were obtained by a BioRad CFX96 Touch™ Real-Time PCR Detection System using SsoAdvanced Universal SYBR Green super Mix (BioRad). The following primers