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Methotrexate administration induces differential and selective protein tyrosine nitration and cysteine nitrosylation in the subcellular organelles of the small intestinal mucosa of rats

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ABSTRACT

Gastrointestinal toxicity is one of the most frequent dose limiting side effects of methotrexate (MTX), a commonly used chemotherapeutic drug. Peroxynitrite (PON) overproduction is reported to contribute to MTX induced gastrointestinal mucositis. However, the consequence of PON overproduction i.e. protein tyrosine nitration and protein cysteine nitrosylation, the subcellular distribution of these modified proteins and their molecular weights have not been investigated yet. Mucositis was induced in Wistar rats by the administration of 3 consecutive i.p. injections of MTX. Tyrosine nitrated proteins and cysteine nitrosylated proteins were determined in the subcellular organelles fractions of mucosa using immunoprecipitation and western blot. The proteins in the subcellular fractions were separated by 1D electrophoresis, and probed with anti -nitrotyrosine antibody and anti-nitrosocysteine antibody. After MTX treatment, a general increase in protein tyrosine nitration as well as a change in the spectrum of proteins that underwent nitration was observed. The relative densities of the 3 nitrotyrosine protein adducts were as follows: Mitochondria > cytosol > microsomes > nucleus. In the mitochondrial fraction increased nitration of 12 kDa, 25 kDa 29Kda, 47 kDa, and 62Kda proteins, in the cytosol increased nitration of 12 kDa, 19 kDa, 45 kDa, and 60 kDa proteins and in the nuclear fraction increased nitration of 17 kDa, 35 kDa, and 58 kDa proteins was observed. On the other hand, MTX treatment resulted to a general decrease in protein cysteine nitrosylation in all the subcellular fractions. These results suggest that MTX induced, PON mediated small intestinal injury is mediated by differential nitration and nitrosylation of proteins in the subcellular organelles with increased protein tyrosine nitration and decreased cysteine nitrosylation. In addition MTX treatment results in selective nitration and nitrosylation of proteins in the intestinal mucosa. This differential nitrosative modifications may contribute to MTX induced small intestinal injury.

1. Introduction

Methotrexate (MTX), a structural analogue of folic acid, is widely used as a chemotherapeutic agent for leukemia and other malignancies [1,2]. It is currently the most common antirheumatic drugs prescribed for the treatment of rheumatoid arthritis and other rheumatic disorders [3]. MTX earned a new indication with its efficacy in the treatment for refractory inflammatory bowel disease [4]. However, the efficacy of MTX is often limited by severe side effects and toxic sequelae.

MTX is transported into the cell via the reduced folate carrier and undergoes polyglutamation catalyzed by folyl-polyglutamate synthetase. Once polyglutamated, MTX is retained in cells for prolonged periods of time. Methotrexate blocks de novo nucleotide synthesis primarily by depleting cells of reduced tetrahydrofolate cofactors through inhibition of dihydrofolate reductase (DHFR) [5]. MTX polyglutamates and dihydrofolates that accumulate as a result of DHFR inhibition also inhibit thymidylate synthase and other enzymes involved in the purine biosynthetic pathway [6]. Following administration of high dose MTX (HDMTX), two metabolites, 7-hydroxy-methotrexate (7-OH-MTX) and 2, 4-diamino-N10-methylpteroic acid (DAMPA), are observed in plasma. 7-OH-MTX is formed by the action of the enzyme aldehyde oxidase on MTX [7]. Intracellular polyglutamation of 7-OH-MTX results in its

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prolonged retention and enhanced cytotoxicity [8].

Gastrointestinal toxicity is one of the most frequently observed side effects that may lead to a reduction in the dose or even a discontinuation of the drug [9,10]. The small intestinal damage induced by MTX treatment results in malabsorption and diarrhoea [11,12]. Approximately 60% of cancer patients that receive a chemotherapy treatment that includes MTX experience diarrhoea [13]. This malabsorption results in weight loss and disturbs the cancer chemotherapy of patients. The mechanism of MTX induced small intestinal injury is not fully understood.

Generation of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) is a cardinal feature of inflamed tissues including those of the gastrointestinal tract. Studies have demonstrated that nitric oxide is an important mediator of chemotherapy induced gastrointestinal mucositis in animal models [14,15]. In normal conditions small amounts of NO produced by endothelial NOS (eNOS) maintains blood supply to the gastrointestinal mucosa. In inflammatory conditions, large amounts (micromolar) of NO are produced by iNOS. During inflammatory reactions when large amounts of NO and superoxide are formed, the combination of both leads to the formation of reactive nitrogen species, such as the peroxynitrite. This toxic compound has the ability to initiate lipid peroxidation, DNA damage, sulfhydryls oxidation, and readily nitrates phenolic compounds such as tyrosine residues on proteins, resulting in augmented inflammation and tissue injury [16]. Because peroxynitrite is impossible to measure *in vivo* owing to its high reactivity, 3-nitrotyrosine formation is used as a fingerprint of peroxynitrite formation. PON nitrates Tyr residues of various proteins generating 3-nitroTyr (3-NT), which is widely accepted as a foot print of peroxynitrite formation [17]. Peroxynitrite induced nitration of tyrosine residues can lead to changes of protein structure and function [18]. Protein nitration typically is indicated in diseases that have an inflammatory component, possibly caused by up-regulation of the inducible NO synthase (iNOS) [19]. Varieties of post-translationally modified nitrated proteins have been shown to accumulate in apoptotic or inflamed tissues [20].

PON can also interact with cysteine residues of proteins resulting in S nitrosylation of cysteine residues comprised within a variety of proteins [21]. Protein S nitrosylation (PSN) is a post translational modification of protein that occurs when PON or nitric oxide reacts with cysteine thiol residue. Protein s-nitrosylation has been demonstrated to be a key modification of cysteine residues under a variety of physiological and pathophysiological conditions [22]. Studies have shown that nitrosylation of proteins have role on processes like signal transduction, host defense, DNA repair, blood pressure control and neurotransmission [23]. PSN can result in loss of protein function or gain of protein function [24].

We and other have demonstrated causative role of NO and PON in MTX induced small intestinal injury [25–27]. Although the participation of nitric oxide and PON in the MTX induced intestinal injury has been shown, the detailed mechanism is not fully understood. The effects of PON on protein tyrosine nitration and cysteine nitrosylation, the subcellular distribution of nitrosated proteins and nitrosative modifications of individual proteins has not been investigated yet to the best of our knowledge.

The specific objectives of the present study were to

1. Find out whether there is any preferential tyrosine nitration and cysteine nitrosylation of proteins in the subcellular organelles of the small intestinal mucosa
2. Identify the molecular weights of proteins that are tyrosine nitrated and cysteine nitrosylated

As intestinal injury induced by MTX in a rodent model mimics the chemotherapy-associated gut damage that occurs in cancer patients [28], using this model, we examined the detailed role of nitrosative stress in MTX induced small intestinal injury.

We show for the first time that MTX treatment results increased protein tyrosine nitration and decreased cysteine nitrosylation in the small intestinal mucosa of rats. We also demonstrate differential and selective protein tyrosine nitration and protein cysteine nitrosylation in the subcellular organelles of the small intestinal mucosa of rats.

2. Materials and methods

2.1. Chemicals

Methotrexate, 4-(2-Hydroxyethyl) piperazine (2-ethanesulfonicacid) (HEPES), hydrogen peroxide, sucrose, bovine serum albumin (BSA), sodium dodecyl sulfate (SDS), mannitol, EGTA, were purchased from Sigma (St. Louis, MO). The primary mouse monoclonal anti-nitrotyrosine antibody obtained from Santa Cruz (Bombay, India) and rabbit monoclonal anti-nitrosocysteine antibody from Sigma. Secondary antibodies (anti-rabbit and anti-mouse IgG conjugated with horse-radish peroxidase) were obtained from Pierce Biotechnology, C.A, USA. Protease inhibitor cocktail (Sigma-Aldrich). Protein A/G Plus – agarose (Santa Cruz). Polyvinylidenedifluoride (PVDF) membrane (pore size 0.45 μm) was obtained from Millipore, India. The Super Sensitive Polymer/HRP/DAB kit was obtained from BioGenex (Chennai, India). ECL dualvue western blotting markers (Amersham). West Dura chemiluminescent substrate for developing western blots was purchased from Thermo Scientific, C.A, USA. All other chemicals used were of the analytical grade.

2.2. Animals

Adult male Wistar rats (150–200 g) were used for the study. They were housed in standard rat cages. All the rats were exposed to 12 h light-dark cycles and allowed access ad libitum to water and rat chow. The experiments done were approved by the institutional animal ethics committee (IAEC) and were in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA), Government of India.

2.3. Animal treatment

Methotrexate was administered to the rats at the concentration of 7 mg/kg body weight intraperitoneally for three consecutive days as described in the literature causing consistent intestinal injury in normal rats [28]. Control animals received an equal volume of the vehicle alone for three consecutive days intraperitoneally.

2.4. Tissue procurement

On the fourth day, the MTX treated rats and control rats were anesthetized with halothane and killed by cervical dislocation after overnight fast. The abdomen of each rat was opened and the entire length of the small intestine was removed, washed with ice cold normal saline. The intestine was cut opened along its anti-mesenteric border longitudinally and mucosa was harvested by gently scraping with a glass slide and used for studies.

2.5. Preparation of intestinal homogenate

A 10% homogenate of the mucosal scrapings was prepared in homogenizing buffer containing 250 mM sucrose, 5 mM HEPES,