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Original article

Oxidative gastric mucosal damage induced by ischemia/reperfusion and the mechanisms of its prevention by carbon monoxide-releasing tricarbonyldichlororuthenium (II) dimer

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ABSTRACT

Endogenous gaseous mediators, such as nitric oxide, hydrogen sulfide or carbon monoxide (CO) are known to exert anti-inflammatory and anti-oxidative activity due to modulation of various molecular pahtways.

Therefore, we aimed to investigate if CO released from tricarbonyldichlororuthenium (II) dimer (CORM-2) prevents gastric mucosa against ischemia/reperfusion (I/R)-induced injury in male Wistar rats. Animals were pretreated i.g. With vehicle (DMSO and saline, 1:10), CORM-2 (1, 5 or 10 mg/kg) or zinc protoporphyrin IX (ZnPP, 10 mg/kg i.p.), the HMOXs inhibitor. In separate series, rats were pretreated with CORM-2 (5 mg/kg) applied in combination with glibenclamide (10 mg/kg i.g.), N^G-nitro-L-arginine (L-NNA, 20 mg/kg i.p.), 1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ, 10 mg/kg i.p.) or indomethacin (5 mg/kg i.p.). I/R-injuries were induced by clamping celiac artery for 30 min (I) followed by removal of the clamp to obtain R for 3 h. The macroscopic and microscopic area of gastric damage, mucus production and protein expression for HMOX-1/Nrf-2 was determined by planimetry, histology and immunohistochemistry, respectively. Gastric mucosal HMOX-1, HMOX-2, COX-1, COX-2, Kir6.1, Sur2, sGC-α1, sGC-α2, iNOS and eNOS mRNA expression was assessed by real-time PCR. COHb in blood and gastric mucosal CO concentration was analyzed by gas chromatography. Serum content of TGF-β1, TGF-β2, TGF-β3, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, TNF-α, IFN-γ, GM-CSF was evaluated using Luminex platform. PGE_2 concentration and 8-hydroxyguanozine (8-OHG) concentration in gastric mucosa was determined by ELISA.

Exposure to I/R induced extensive hemorrhagic erosions in gastric mucosa pretreated with vehicle as compared with intact rats and the area of this gastric damage was reduced by pretreatment with CORM-2 (5 mg/kg i.g.). This effect of CO donor was accompanied by the increased PGE_2 content and a significant decrease in 8-OHG and expression of pro- and anti-inflammatory markers mRNA and proteins. Concurrent treatment of CORM-2 with glibenclamide, L-NNA, ODQ but not with indomethacin significantly increased the area of I/R-induced injury and significantly decreased GBF as compared with the group treated with CORM-2 alone.

We conclude that CO releasing CORM-2 prevents gastric mucosal oxidative damage induced by I/R improving GBF, decreasing DNA oxidation and inflammatory response on systemic level. This CO-gastroprotection is mediated by the activity of sGC, NOS and K-ATP channels. CO delivered from its donor maintained physiological gastric mucosal PGE_2 concentration but the involvement of endogenous COX in beneficial activity of this gaseous mediator at least in this model is questionable.

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1. Introduction

Recent data has shown that endogenous gaseous mediators, such as hydrogen sulfide (H_2S) or carbon monoxide (CO) are involved, similarly to nitric oxide (NO) in the maintenance of gastric mucosal integrity and in the regulation of gastric microcirculation [1–4]. These gas molecules modulate many cellular pathways and exert anti-inflammatory or anti-oxidative properties as it has been demonstrated under experimental conditions in various models of gastrointestinal (GI) pathologies, such as drugs-induced gastrotoxicity, peptic ulcer healing, stress-induced gastric damage or experimental colitis [1–3,5–7]. Interestingly, it has been demonstrated that CO released from its pharmacological donor, tricarbonyldichlororuthenium (II) dimer (CORM-2) can accelerate healing of chronic gastric ulcers, possibly due to modulation of various molecular pathways and growth factors but independently on endogenous H_2S biosynthesis [7]. Importantly, CO is produced endogenously *via* heme degradation by the activity of inducible heme oxygenase 1 (HMOX-1) and constitutive HMOX-2 [3].

Previous studies demonstrated that CO produced by HMOX or released from appropriate pharmacological donors has been shown to ameliorate injury caused by renal, cerebral and myocardial ischemia/reperfusion (I/R) [8–11]. However, possible gastroprotective effect and mechanisms of CO releasing from pharmacological donors against I/R-induced gastric damage has not been fully explained. Therefore, we aimed to investigate if endogenously produced CO *via* HMOX-1/Nuclear factor (erythroid-derived 2)-like 2 (Nrf-2) or pretreatment with CO releasing CORM-2 modulates gastric blood flow (GBF) and can protect the gastric mucosa from gastric lesions induced by I/R and DNA oxidation on macroscopic, microscopic, biochemical and molecular levels. We focused on the possible involvement of gastroprotective factors such as endogenous prostaglandin E_2 (PGE_2) produced by enzymatic activity of 1) cyclooxygenase (COX)-1 and COX-2, 2) ATP-dependent potassium channels (K-ATP channels), 3) soluble guanylyl cyclase (sGC) and inducible or endothelial NO synthases, iNOS and eNOS, respectively, as possible mediators of CO-induced gastroprotection against I/R-induced gastric damage. Furthermore, we assessed alterations in gastric mucosal content of CO and COHb concentration in blood of rats exposed to gastric I/R with or without CORM-2 pretreatment. We have also determined the contribution of anti-inflammatory properties of CO in its gastroprotective action against I/R-injury by screening of the serum concentration profile of 14 pro- and anti-inflammatory factors.

2. Materials and methods

2.1. Experimental design: animals, chemicals and drugs, I/R-injury

Male Wistar rats in the number of 60, with average weight 250–300 g were fasted for 24 h before the experiments with free access to drinking water. The study was approved by the Institutional Animal Care and Use Committee of Jagiellonian University Medical College in Cracow and conducted in accordance with Helsinki Declaration regarding handling of experimental animals (Approval no 79/2017, permission date: 19 July 2017) and run in accordance with the statements of the Helsinki Declaration regarding handling of experimental animals. Experiments were run with implications for replacement, refinement or reduction (the 3Rs) principle. Animal studies are reported in compliance with the ARRIVE guidelines.

All chemicals and drugs used for in vivo experiments and molecular or biochemical assessments were purchased from Sigma-Aldrich (Schnelldorf, Germany) unless otherwise stated.

In the day of experiment, animals of series A were randomized to the appropriate experimental groups treated i.g. With 1) DMSO and saline (1:10) as vehicle, 2) CO releasing CORM-2 [7,12], applied in various doses: 1, 5 or 10 mg/kg or 3) zinc protoporphyrin IX (ZnPP), the HMOXs inhibitor [13], administered i.p. In a dose previously reported in our studies to decrease CO content in gastric mucosa [14]. In separate series B, rats were pretreated with CORM-2 alone administered in a dose of 5 mg/kg, selected as the most effective in series An experiments, applied in combination with 1) glibenclamide (10 mg/kg i.g.), inhibitor of K-ATP channels [15], 2) N^G-nitro-L-arginine (L-NNA, 20 mg/kg i.p.), non-selective NO synthase (NOS) inhibitor [16], 3) 1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ, 10 mg/kg i.p.), sGC inhibitor [17,18] or 4) indomethacin (5 mg/kg i.p.), non-selective COX inhibitor [18]. All compounds were injected i.p. or administered i.g. using orogastric tube as reported previously [14]. To decrease the impact of too many confounding factors provided directly to the gastric mucosa possibly affecting the effectiveness of experimental model, some agents were applied i.p. If they were originally reported to maintain its effectiveness on gastric mucosa when administered by this route [14,16–18]. Exceptionally, glibenclamide was applied i.g. following previously described protocol and because based on previous reports this route did not affect its effectiveness and it even required much lower doses of the drug when applied i.g. to evoke desired experimental effects [15,18–20].

Acute I/R gastric lesions were induced 30 min after treatments, as described previously [21,22]. Briefly, under isofluorane anesthesia, the abdomen was opened, and the celiac artery was identified and clamped for 30 min of ischemia (I) followed by removal of the clamp to obtain reperfusion (R) for 3 h.

2.2. Macroscopic assessment of gastric damage area and determination of GBF level, biological samples collection

After 3 h of R, under isofluorane anesthesia the area of gastric damage was measured by planimetry and the GBF was determined by laser flowmetry as described previously [18]. Briefly, the GBF was measured in oxyntic part of the gastric mucosa not involving I/R mucosal lesions using laser flowmeter (Laserflo, model BPM 403A, Blood Perfusion Monitor, Vasamedics, St. Paul, Minnesota, USA). Average values of three measurements were determined and expressed in mL/min per 100 g of gastric tissue. Blood was collected from *vena cava* and whole blood or serum samples were stored in −20 °C for further analyses. The area of I/R-induced gastric damage in each rat stomach was determined planimetrically and expressed in mm^2 [22]. Rats were sacrificed by i.p. administration of lethal dose of pentobarbital (Biowet, Pulawy, Poland). Next, the gastric mucosal samples from oxyntic mucosa were scraped off on ice, snap-frozen in liquid nitrogen and stored at −80 °C until further analysis [18].

2.3. Microscopic assessment of I/R gastric damage, mucus production and Nrf-2 and HMOX-1 protein distribution

For histology, the gastric tissue sections were excised and fixed in 10% buffered formalin, pH 7.4. Samples were dehydrated by passing them through a series of alcohols with incremental concentrations, equilibrated in xylene for 10–15 min and embedded in paraffin; paraffin blocks were cut into about 4 μm sections using a microtome. The prepared specimens were stained with haematoxylin/eosin (H&E) or alcian blue/periodic acid-Schiff/alcian blue (AB/PAS) [23].

To evaluate gastric mucosal Nrf-2 and HMOX-1 proteins distribution within gastric mucosa immunohistochemistry was employed. Endogenous peroxidase was blocked by 3% H_2O_2 solution in above-mentioned specimens and incubated in waterbath in the presence of EDTA or sodium citrate and treated with proteinase K for 7 min. Next, tissue slides were stained for 30 min with anti-HMOX-1 mouse monoclonal antibody (1:500; 66743-1-Ig; Proteintech, Manchester, The United Kingdom) and Nrf-2 rabbit polyclonal antibody (1:200; 16396-1-AP; Proteintech). BrightVision plus Poly-HRP-Anti MS/Rb IgG system containing HRP-linked secondary antibody was further implemented. Next, DAB Quanto (TA-125-QHDX; Thermo Fisher Scientific, Waltham, MA, USA) was used to visualize investigated proteins in gastric mucosal tissue sections.

Samples were evaluated using a light microscope (AxioVert A1, Carl Zeiss, Oberkochen, Germany) [23]. Digital documentation of histological slides was obtained using above mentioned microscope equipped with automatic scanning table and ZEN Pro 2.3 software (Carl Zeiss, Oberkochen, Germany) to collect multiple photographs of each histological sample and to stitch them into one picture; to obtain better quality of each picture, the background was subtracted and unified as white [23].

2.4. Determination of CO content in gastric mucosa and blood carboxyhemoglobin (COHb) concentration using gas chromatography (GC)

CO concentrations in the gastric mucosa biopsies and COHb levels in whole blood samples were determined as reported previously but using modified GC based method described in details elsewhere and briefly below [14,24].

Samples preparation: 10 ml of water were added to the tissue fragments (about 400–600 mg) and to the 400 µl of blood sample, 9.6 ml of water and 50 mg of sodium thiosulfate were added. Both samples were homogenized by sonication (Bandelin Sonoplus, Germany). The volume of 2.5 ml of homogenate were pipetted into two 10 ml headspace vials (2 test samples). To obtain calibration samples, about 5 ml of the remaining volume of the homogenate was saturated with CO for 20 min (100% saturation CO). The CO used to saturate the calibration samples was obtained by reacting concentrated sulfuric acid with 80% formic acid. Unbound CO was removed by flushing with nitrogen for 3 min. Calibration solutions with CO saturation 1.25; 2.5; 5 and 10% were prepared from 100% saturated homogenates. 2.5 ml of each calibration solution was pipetted into headspace vials (4 calibration samples). The vials were then sealed with an aluminum cap and silicon/Teflon septum. Each vial was gently flushed with helium for 30 s and then 1.5 ml of 20% potassium hexacyanoferridate was added with a syringe.

GC/O-FID-headspace analysis: for the GC/O-FID-headspace analysis a Thermo Trace GC Ultra (Thermo Electron Corp. USA) equipped with O-FID detector (FID with jet nickel microcatalytic methanizer) was used. The jet nickel microcatalyzer converts CO to methane at 330 °C which increases the sensitivity of CO detection. The system was equipped with Thermo TriPlus HS autosampler. The prepared samples were mixed and incubated at 70 °C for 8 min in autosampler agitator to the achieve a complete CO liberation. 200 µL of gas-phase of each sample were injected with an autosampler gas-tight syringe (heated at 72 °C). Split/splitess injector (200 °C) with closed split was used. GC separation was performed with HP-Molesieve column (Agilent Technologies, USA) 30 m/0.53 mm ID/0,25 µm at constant flow 15 ml/min of helium as a carrier gas. The temperature program consisted of the following steps: 60 °C for 2 min followed by 120 °C for 2 min achieved by a heating rate 60 °C/min.

2.5. Determination of mRNA expression for HMOX-1, HMOX-2, COX-1, COX-2, Kir6.1, Sur2, sGC-α1, sGC-α2, iNOS and eNOS in gastric mucosa by real-time polymerase chain reaction (PCR)

Gastric mucosal mRNA expression for HMOX-1, HMOX-2, COX-1, COX-2, Kir6.1, Sur2, sGC-α1, sGC-α2, iNOS and eNOS was determined by real time PCR, as described previously [23]. Total RNAwas isolated using commercially available kit with spin columns (GeneMATRIX Universal RNA Purification Kit, EURx, Gdansk, Poland) according to manufacturer protocol. Reversed transcription to cDNA was performed using High-Capacity cDNA Reverse Transcription Kit (MultiScribe™, Applied Biosystems, Life Technologies, Carlsbad, CA, USA) and random primers.

RNA concentration was measured using Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). RT was normalized for each reaction regarding total RNA concentration to obtain the same value (2 µg) for each sample. Results obtained for RNA samples isolated from healthy (intact) gastric mucosa and transcribed to cDNA were further used as reference control during calculations.

Expression for HMOX-1, HMOX-2, COX-1, COX-2, iNOS, eNOS and B-actin and succinate dehydrogenase complex (SDHA) as reference genes was determined using specific primers [23]. Kir6.1 was determined using 5′- TGGAGAAAGGCATCACGGAG-3′ forward and 5′-AACGCAGAAGTGAATGACCTGA-3′ reverse primers. Sur2 was determined using 5′-GCCATAAAGGTGACGAACGG-3′ forward and 5′-GACGATTTGCCACAACCCAC-3′ reverse primers. sGC-α1 was determined using 5′- TCATCACCATGCTCAACGCT-3′ forward and 5′-TCCGATGGTCTCCACCTTGT-3′ reverse primers. sGC-α2 was determined using 5′-GTTGCATCAGGGCTTCACAG-3′ forward and 5′-ATC CGCATCTGAATGGGTCTT-3′ reverse primers.

Real-time PCR was conducted using thermal cycler Quant Studio 3 (Thermo Fisher Scientific, Waltham, MA, USA) and SYBR Green dye including kit (SG qPCR Master Mix (2x), EURx, Gdansk, Poland). Results were analyzed using ΔΔCt method [23,25].

2.6. Measurement of serum content of pro- and anti-inflammatory factors by luminex microbeads fluorescent assays

Determination of TGF-β1, TGF-β2, TGF-β3, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, TNF-α, IFN-γ, GM-CSF concentrations in serum was performed using Luminex micro beads fluorescent assays (Bio-Rad, California, USA) and Luminex MAGPIX System (Luminex Corp., Austin, TX, USA). Results were calculated from calibration curves and expressed in pg/ml, according to the manufacturers protocol, as described previously [7].

2.7. Determination of PGE_2 and 8-hydroxyguanozine (8-OHG) concentration in gastric mucosa

PGE_2 concentration in gastric mucosal samples obtained from ulcer margin was determined using PGE2 ELISA kit (ab133021, Abcam) according to manufacturer's protocol. Homogenization process of each sample was standarized regarding sample weight and buffer volume and results were expressed in pg/ml of gastric tissue homogenate. 8-OHG content as DNA oxidation marker was assessed in DNA isolated from gastric mucosa using ELISA kit (589320, Cayman Chemical, Ann Arbor, MI, USA) according to manufacturer's protocol.

2.8. Statistical analysis

Experiments and data collection were done by operators blinded to the sample identity. Results were analyzed using GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA). Results are presented as mean ± SEM. Statistical analysis was conducted using Student's t-test or ANOVA with Dunnett's multiple comparison or Tukey's post hoc test if more than two experimental groups were compared. The size for each experimental group was of n = 5–6. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Gastroprotective and hyperemic effects of CO releasing CORM-2 against I/R-induced gastric damage, mucus production and modulation of HMOX-1 and Nrf-2 protein distribution

Fig. 1A and B shows the mean area of I/R-induced gastric damage and changes in GBF, respectively, in rats pretreated with vehicle, CORM-2 (1–10 mg/kg i.g.) or ZnPP (10 mg/kg i.p.). CORM-2 administered in a dose of 1 mg/kg or 5 mg/kg but not 10 mg/kg, significantly decreased the area of I/R gastric damage and significantly increased GBF as compared with vehicle-treated animals ($p < 0.05$) (Fig. 1A–B). Pretreatment with ZnPP did not affect significantly mean gastric damage area and GBF as compared with vehicle (Fig. 1A and B).

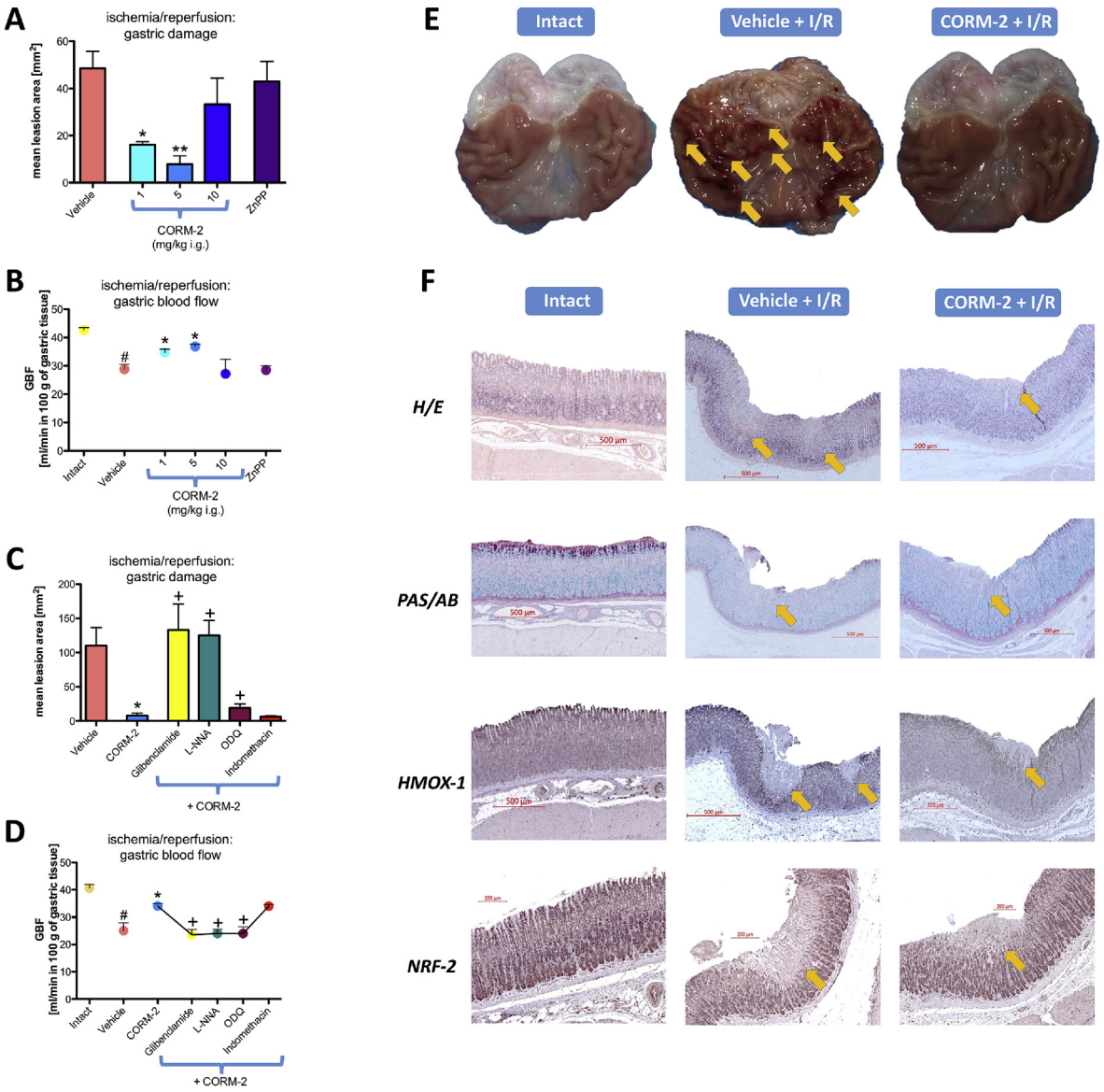

Fig. 1. Mean lesion area (A, C) and the gastric blood flow (GBF) (B, D) in intact (control) gastric mucosa and in gastric mucosa of rats pretreated with vehicle or tricarbonyldichlororuthenium(II) dimer (CORM-2, 5 mg/kg i.g.) alone or in combination with glibenclamide (10 mg/kg i.g.), N^G-nitro-L-arginine (L-NNA, 20 mg/kg i.p.), 1H- [1,2,4]oxadiazolo [4,3-a]quinoxalin-1-one (ODQ, 10 mg/kg i.p.), indomethacin (5 mg/kg i.p.) and exposed to 3.5 h of ischemia/reperfusion (I/R). Intact refers to healthy gastric mucosa not exposed to I/R. ZnPP refers to the group pretreated with zinc protoporphyrin IX (10 mg/kg i.p.) and exposed to I/R. Results are mean ± S.E.M of 5 rats per each experimental group. Significant change as compared with the respective values in vehicle-control group is indicated by asterisk ($p < 0.05$). Double asterisk indicates significant change as compared with the respective values in rats pretreated with vehicle and CORM-2 administered in a dose of 1 mg/kg ($p < 0.05$). Cross indicates significant change as compared with the respective values in rats pretreated with CORM-2 alone ($p < 0.05$). Significant change as compared with the GBF values in intact rats is indicated by hash ($p < 0.05$). E: Macroscopic appearance of randomly selected representative gastric mucosa exposed or not to I/R; gastric mucosal injuries pointed by yellow arrows. F: Randomly selected representative histological slides of gastric mucosa exposed or not to I/R with or without vehicle or CORM-2 pretreatment and stained with H/E, PAS/AB or anti-HMOX-1 or anti-Nrf-2 antibodies; gastric injuries pointed by yellow arrows.

Pretreatment with CORM-2 combined with glibenclamide (10 mg/kg i.g.), L-NNA (20 mg/kg i.p.) or ODQ (10 mg/kg i.p.) but not with indomethacin (5 mg/kg i.p.) significantly increased the area of gastric I/R lesions and significantly decreased GBF as compared with rats treated with CORM-2 alone ($p < 0.05$) (Fig. 1C and D). When ODQ was co-administered with CORM-2 a significant decrease in area of gastric I/R damage as compared with the vehicle-treated animals was observed ($p < 0.05$) (Fig. 1C).

As shown in Fig. 1E, the exposure of gastric mucosa to I/R induced extensive hemorrhagic erosions in gastric mucosa pretreated with vehicle (arrows) as compared with intact rats and the area of this lesions was significantly reduced in rats pretreated with CORM-2 (5 mg/kg i.g.) *vs* vehicle. Fig. 1F shows representative histology photomicrographs of intact (control) gastric mucosa and gastric mucosa pretreated with vehicle or CORM-2 (5 mg/kg i.g.) and exposed to I/R. Alterations in microscopic appearance, mucus production, HMOX-1 and Nrf-2 protein expression in gastric mucosa was demonstrated using H/E, AB/PAS and IHC staining, respectively (Fig. 1F). I/R induced necrotic erosions of surface epithelium penetrating deeply into gastric mucosa reaching lamina propria with submucosal leukocytes infiltration as compared with intact rats (intact vs vehicle; H/E; Fig. 1F). These alterations were accompanied by the disruption of mucus layer of surface epithelium and decreased yield of HMOX-1 and Nrf-2 expression within damaged part of gastric mucosa (intact vs vehicle; AB/PAS, HMOX-1, Nrf-2; Fig. 1F). Pretreatment with CORM-2 limited the histologic injury of I/R-induced erosions to the superficial epithelium but failed to affect the

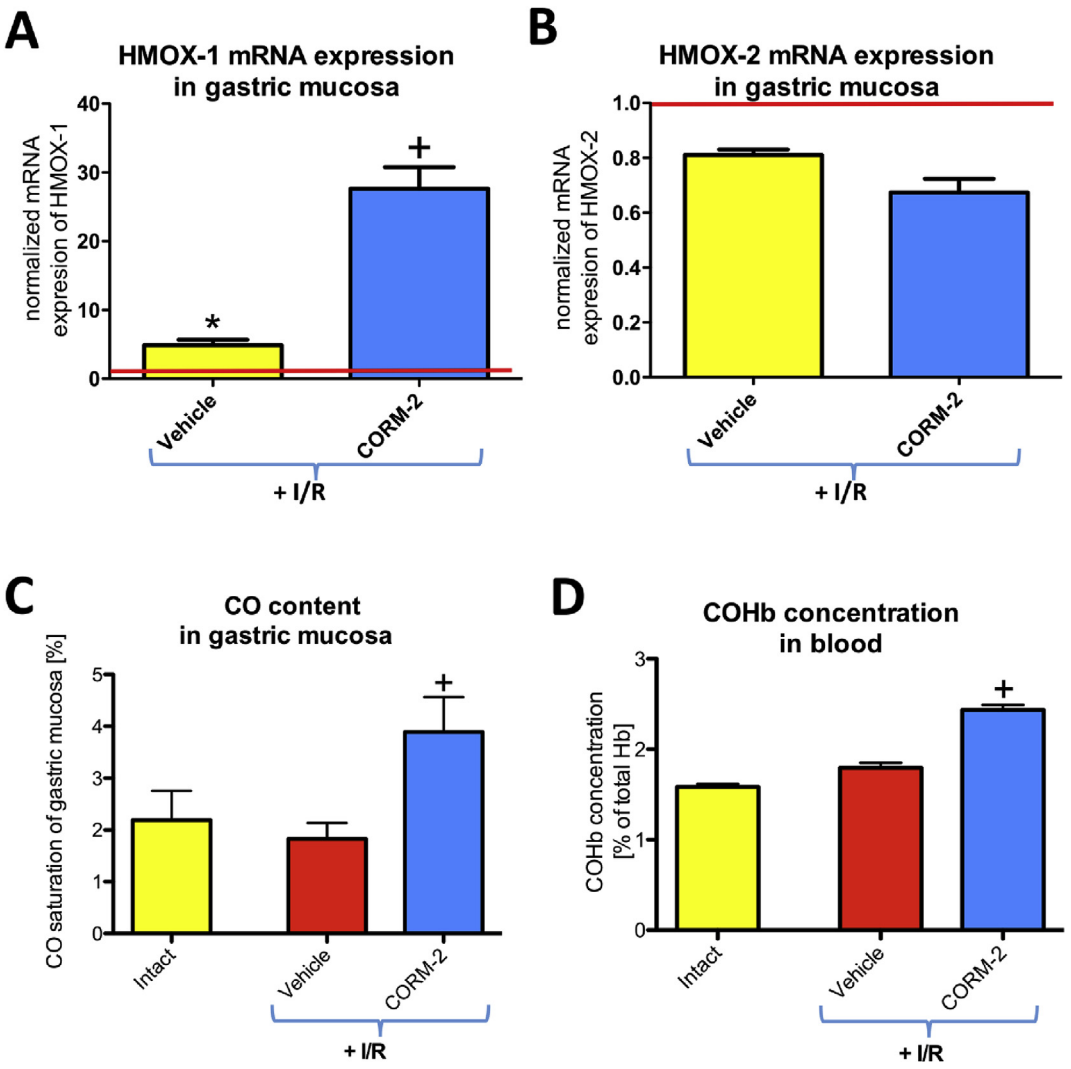

Fig. 2. Heme oxygenase (HMOX)-1 and 2 mRNA expression and CO saturation in gastric mucosa and the concentration COHb in blood of rats pretreated with vehicle or tricarbonyldichlororuthenium(II) dimer (CORM-2, 5 mg/kg i.g.) and exposed to 3.5 h of ischemia/reperfusion (I/R). Intact refers to healthy gastric mucosa not exposed to I/R. Results are mean ± S.E.M of 5 samples per each experimental group. A and B: results are expressed as fold change of normalized gastric mucosal HMOX-1 and HMOX-2 mRNA expression; red line refers to the baseline expression in intact gastric mucosa. C and D: results are expressed as % of CO saturation of gastric mucosa or COHb concentration in blood, respectively. Asterisk indicates a significant change as compared with respective values obtained in intact group ($p < 0.05$). Cross indicates a significant change as compared with the values obtained in the vehicle-pretreated group exposed to I/R ($p < 0.05$). A and B: statistical significance was marked only if 2-fold up- or downregulation was reached.

decrease in mucus production and HMOX-1 and Nrf-2 expression within these injurious sites of gastric mucosa (vehicle vs CORM-2; H/E, AB/PAS, HMOX-1, Nrf-2; Fig. 1F).

3.2. Alterations in HMOX-1, HMOX-2 mRNA expression and in CO content in gastric mucosa and blood COHb concentration of rats exposed to I/R and pretreated or not with CORM-2

Fig. 2A–B shows that HMOX-1 (A) but not HMOX-2 (B) mRNA expression in gastric mucosa with I/R-induced gastric damage was significantly increased as compared with the respective values in intact gastric mucosa ($p < 0.05$) (Fig. 2A–B). Pretreatment with CORM-2 significantly increased mRNA expression of HMOX-1 but not HMOX-2 as compared with the vehicle-control group ($p < 0.05$) (Fig. 2A–B).

CO saturation of gastric mucosa with I/R-induced gastric damage was not significantly affected as compared with intact gastric mucosa ($p < 0.05$) (Fig. 2C). Pretreatment with CORM-2 significantly increased gastric mucosal CO content as compared with the vehicle-control group ($p < 0.05$) (Fig. 2C). The blood COHb concentration was not significantly affected in rats with I/R-induced gastric damage as compared with intact animals ($p < 0.05$) (Fig. 2D). Pretreatment with CORM-2 significantly increased blood concentration of COHb as compared with the vehicle-control group ($p < 0.05$) (Fig. 2D).

3.3. The changes in COX-1 and COX-2 mRNA expression and PGE_2 content in gastric mucosa exposed to I/R and pretreated or not with CORM-2

Fig. 3A–B shows that COX-1 (A) and COX-2 (B) mRNA expression in gastric mucosa with I/R-induced gastric damage was not significantly affected as compared with the respective values in intact gastric mucosa (Fig. 3A–B). Pretreatment with CORM-2 did not affect mRNA expression of COX-1 or COX-2 as compared with the vehicle-control group (Fig. 3A–B).

PGE_2 concentration in gastric mucosa with I/R-induced gastric damage was significantly decreased as compared with intact gastric mucosa ($p < 0.05$) (Fig. 3C). Pretreatment with CORM-2 significantly increased gastric mucosal PGE_2 content as compared with the vehicle-control group ($p < 0.05$) (Fig. 3C).

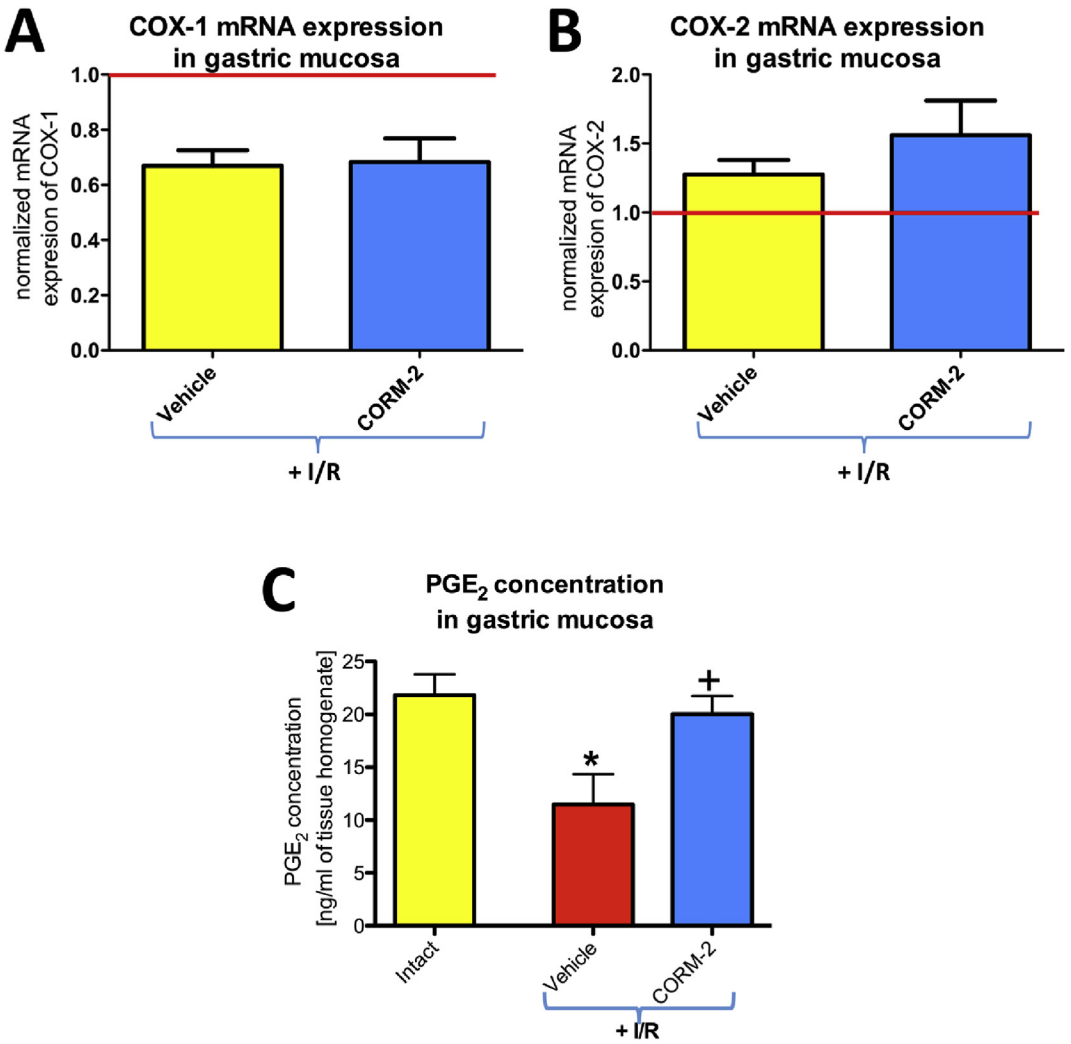

Fig. 3. Cyclooxygenase (COX)-1 and COX-2 mRNA expression in gastric mucosa of rats pretreated with vehicle or tricarbonyldichlororuthenium(II) dimer (CORM-2, 5 mg/kg i.g.) and exposed to 3.5 h of ischemia/reperfusion (I/R). Intact refers to healthy gastric mucosa not exposed to I/R. Results are mean ± S.E.M of 5 samples per each experimental group. A and B: results are expressed as fold change of normalized gastric mucosal COX-1 and COX-2 mRNA expression; red line refers to the baseline expression in intact gastric mucosa. A and B: statistical significance was marked only if 2-fold up- or downregulation was reached. C: results are expressed as PGE_2 level in gastric mucosa. Asterisk indicates a significant change as compared with respective values obtained in intact group ($p < 0.05$). Cross indicates a significant change as compared with the values obtained in the vehicle-pretreated group exposed to I/R ($p < 0.05$).

3.4. Alterations in mRNA expression of K-ATP channels subunits, Kir6.1 and Sur2 and sGC-α1 and α2 subunits in gastric mucosa exposed to I/R with or without pretreatment with CORM-2

Fig. 4 shows that Kir6.1 (A) and Sur2 (B) mRNA expression in gastric mucosa with I/R-induced gastric damage was significantly downregulated as compared with the respective values in intact gastric mucosa ($p < 0.05$) (Fig. 4A–B). Pretreatment with CORM-2 did not affect mRNA expression of Kir6.1 or Sur2 as compared with the vehicle-control group (Fig. 4A–B). Fig. 4 shows that sGC-α1 (C) and α2 (D) mRNA expression in gastric mucosa with I/R-induced gastric damage was significantly downregulated as compared with the respective values in intact gastric mucosa ($p < 0.05$) (Fig. 4C–D). Pretreatment with CORM-2 did not affect mRNA expression of Kir6.1 or Sur2 as compared with the vehicle-control group (Fig. 4C–D).

3.5. The effect of pretreatment with CORM-2 on mRNA expression of NOS isoforms, iNOS and eNOS in gastric mucosa exposed to I/R

Fig. 5 shows that iNOS (A) but not eNOS (B) mRNA expression was significantly upregulated in gastric mucosa with I/R-induced gastric damage as compared with the respective values in intact gastric mucosa ($p < 0.05$) (Fig. 5A and B). Pretreatment with CORM-2 significantly decreased mRNA expression of iNOS (A) but not eNOS (B) as compared with the vehicle-control group ($p < 0.05$) (Fig. 5A and B).

3.6. Oxidative DNA damage in gastric mucosa exposed to I/R pretreated with vehicle or CORM-2

Fig. 6 shows that 8-OHG content was significantly upregulated in gastric mucosa with I/R-induced gastric damage as compared with the respective values in intact gastric mucosa ($p < 0.05$). Pretreatment with CORM-2 significantly decreased 8-OHG content as compared with the vehicle-control group ($p < 0.05$) (Fig. 6).

3.7. The effect of pretreatment with vehicle or CORM-2 on pro- and anti-inflammatory cytokines concentration in serum of rats exposed to I/R

Fig. 7A-N shows that exposure to I/R significantly increased concentration of TGF-β2 (B), TGF-β3 (C), IL-1α (D), IL-1β (E), IL-2 (F), IL-4 (G), IL-5 (H), IL-6 (I), IL-10 (J), IL-12 (K), TNF-α (L), IFN-γ (M), GM-CSF (N) but not TGF-β1 (A) in serum as compared with intact rats

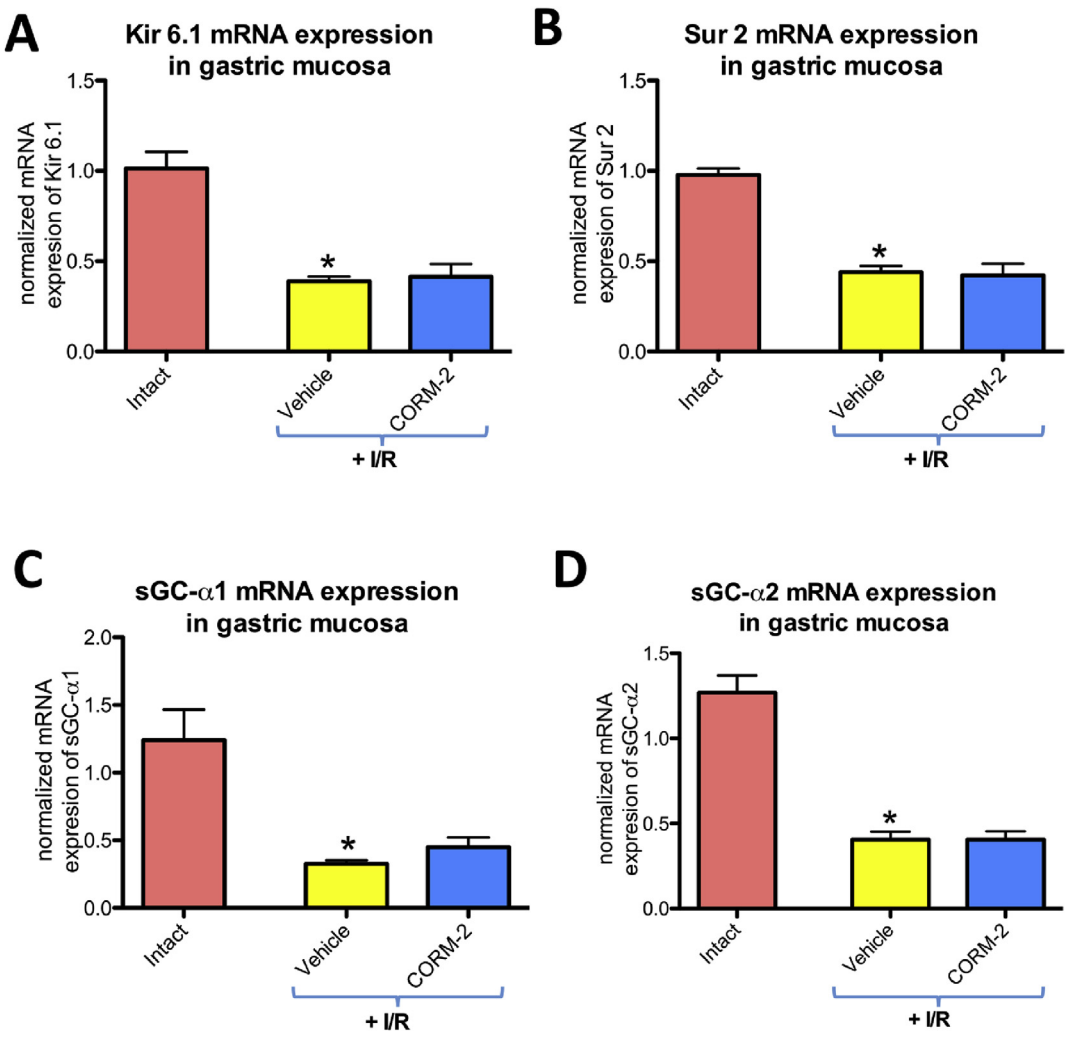

Fig. 4. Gastric mucosal mRNA expression of ATP-dependent potassium channels subunits Kir6.1 (A) and Sur2 (B) and soluble guanylyl cyclase (sGC)-α1 (C) and sGC-α2 (D) subunits, determined in rats pretreated with vehicle or tricarbonyldichlororuthenium(II) dimer (CORM-2, 5 mg/kg i.g.) and exposed to 3.5 h of ischemia/reperfusion (I/R). Intact refers to healthy gastric mucosa not exposed to I/R. Results are mean ± S.E.M of 5 samples per each experimental group with statistical significance marked only if 2-fold up- or downregulation was reached. Results are expressed as fold change of normalized gastric mucosal Kir6.1, Sur2, sGC-α1 and sGC-α2 mRNA expression. Asterisk indicates a significant change as compared with respective values obtained in intact group ($p < 0.05$).

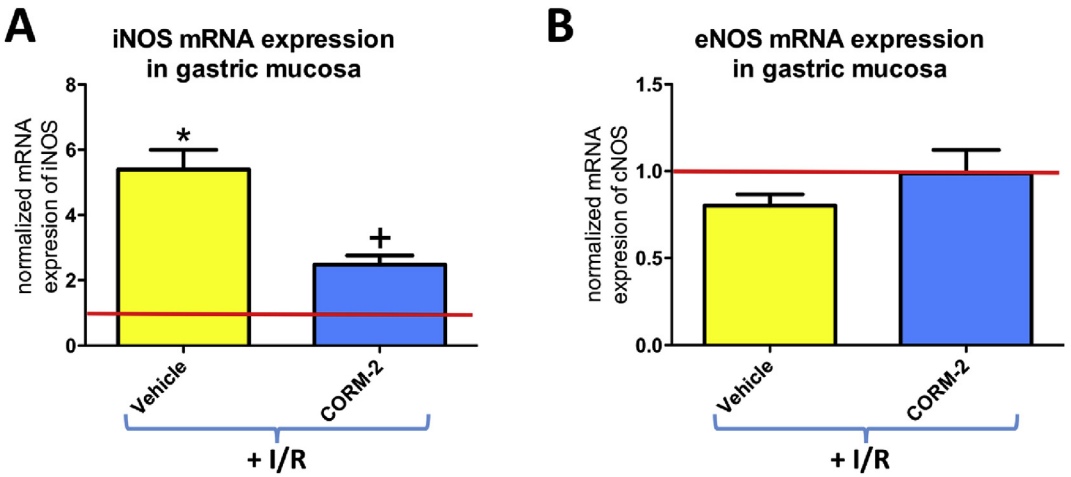

Fig. 5. Expression of iNOS (A) and eNOS (B) mRNA in gastric mucosa of rats pretreated with vehicle or tricarbonyldichlororuthenium(II) dimer (CORM-2, 5 mg/kg i.g.) and exposed to 3.5 h of ischemia/reperfusion (I/R). Results are mean ± S.E.M of 5 samples per each experimental group with statistical significance marked only if 2-fold up- or downregulation was reached. Results are expressed as fold change of normalized gastric mucosal iNOS and eNOS mRNA expression. Asterisk indicates a significant change as compared with respective values obtained in intact gastric mucosa, not exposed to I/R ($p < 0.05$). Cross indicates a significant change as compared with the values obtained in the vehicle-pretreated group exposed to I/R ($p < 0.05$).

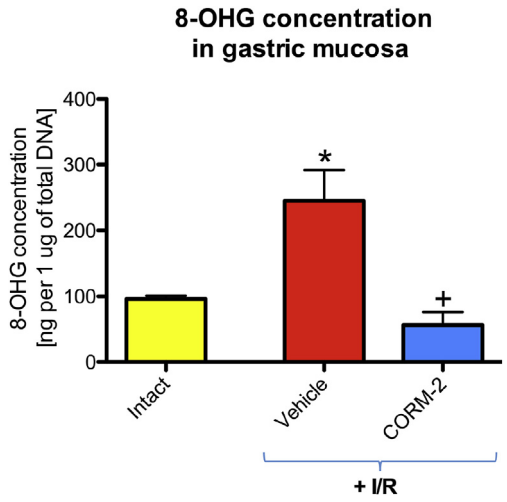

Fig. 6. Gastric mucosal 8-hydroxyguanozine (8-OHG) concentration determined in rats pretreated with vehicle or tricarbonyldichlororuthenium(II) dimer (CORM-2, 5 mg/kg i.g.) and exposed to 3.5 h of ischemia/reperfusion (I/R). Intact refers to healthy gastric mucosa not exposed to I/R. Results are mean ± S.E.M of 5 samples per each experimental group. Results are expressed as 8-OHG concentration standarized to 1 μg of total DNA per each sample. Asterisk indicates a significant change as compared with respective values obtained in intact group ($p < 0.05$). Cross indicates a significant change as compared with respective values obtained in group pretreated with vehicle and exposed to I/R ($p < 0.05$).

($p < 0.05$). Pretreatment with CORM-2 significantly decreased TGF-β3 (C), IL-1α (D), IL-1β (E), IL-2 (F), IL-4 (G), IL-5 (H), IL-6 (I), IL-10 (J), IL-12 (K), TNF- α (L), IFN-γ (M), GM-CSF (N) but not TGF-β1 (A) or TGF-β2 (B) concentrations in serum or rats exposed to I/R as compared with vehicle-control group ($p < 0.05$) (Fig. 7A-N).

4. Discussion

Previous studies have demonstrated that the exposure to I/R led to induction of reactive oxygen species (ROS) generation from xanthine–xanthine oxidase system and an increase in tissue lipid peroxidation due to activated neutrophils, resulting in gastric mucosal hemorrhagic lesions [26–29]. We have documented in this study that pretreatment with CORM-2 increased gastric mucosal CO content, raised GBF and the mRNA expression for HMOX-1 resulting in gastroprotection against the formation of I/R-induced gastric damage and DNA oxidation. Moreover, we observed that exposure to I/R upregulated gastric mucosal mRNA expression of HMOX-1, involved in endogenous production of CO. However, within I/R-injured parts of gastric epithelium, the expression of HMOX-1 and Nrf-2 proteins were not detectable. This is in pair with previously published data showing that HMOX-1 mRNA expression was increased in various experimental models of oxidative and inflammatory injury [3]. Additionally, it has been shown previously that administration of CORM-2 under pathological conditions even enhanced HMOX-1 mRNA expression [14,23,30], suggesting that this enhancement could be due to positive feedback mechanism. Possibly, pretreatment with CO donor enhanced self-defence gastric mucosal response against I/R injury manifested as upregulation of HMOX-1 mRNA expression.

Interestingly, gastroprotective effect of CORM-2 was dose-dependent to certain degree because when applied in a dose of 10 mg/kg this CO-donor did not exert protection against I/R-damage. Indeed, previous report documented that pretreatment with CO donor administered i.g. In higher dose, 50 mg/kg exacerbated ethanol-induced gastric mucosal erosions [30]. This suggests that increased bioavailability of CO is beneficial to gastric mucosa but when a threshold concentration of this gas in the tissue is exceeded, its cytotoxicity under specific pathological conditions can occur. Our finding on CO-induced gastroprotection against I/R injury paralleled our previous observations that CO-releasing chemicals prevented stress-induced gastric lesions and those evoked by i.g. administration of ethanol or alendronates [14,23,31–34]. Our present data in experimental model of I/R is corroborative with previous observations that HMOX-1 and CO releasing CORM-2 or CORM-3 ameliorated I/R-induced damage to the liver or skeletal muscles [35–38].

I/R-injuries were shown to progress with longer time into chronic ulcerations [39]. It has been previously reported that CORM-2-mediated healing of chronic gastric ulcers was diminished by combined treatment of this CO-donor with non-selective COX-1 and COX-2 inhibitor, indomethacin, administered daily through 9 days after ulcer induction [18]. Interestingly, in our study we have observed that I/R-induced decrease in mucosal PGE_2 content was reversed by pretreatment with this CO donor. However, the mRNA expression of COX-1 or COX-2 was not affected in gastric mucosa with or without pretreatment with CORM-2. Furthermore, CO donor was still effective in protection of gastric mucosa against I/R-damage despite the presence of indomethacin. Therefore, we assume that CO released from CORM-2 could bind to heme domain and as result, it could affect COX activity even during exposure to I/R, thus maintaining the gastric mucosal PGE_2 production. However, at least in this model of gastric injury, CO seems to exert its gastroprotective activity independently on PGE/COX system.

We have demonstrated that pharmacological inhibition of K-ATP channels by glibenclamide completely reversed CORM-2-mediated gastric hyperemia and gastroprotection against I/R injury. Moreover, pretreatment with CO donor did not affect decreased mRNA expression for K-ATP channels subunits, Kir6.1 and Sur2. This is corroborative with previous observation that glibenclamide decreased CO effect on Cl^- and HCO_3^- anions secretion in rats colon tissue [40]. Furthermore, Soni et al. revealed that CORM-2 reduces I/R injury in Langendorff's perfused rat hearts and that this cardioprotective effect of CO donor was reversed by K-ATP channels inhibition [41]. Therefore, we assume that CO-mediated protection against I/R injuries involves K-ATP channels activity, possibly due to activation of heme binding domain Sur 2A by this gaseous mediator [42].

Furthermore, the pharmacological inhibition of NOS and sGC activity also reversed and reduced, respectively CORM-2-mediated gastroprotection against I/R injury and accompanying elevation of GBF. This is in pair with previously published data showing that ulcer healing effect of CO donor was attenuated by NOS blockade by L-NNA and inhibition of sGC activity by ODQ [18]. Interestingly, in this study we have observed that pretreatment with CORM-2 remained without effect on gastric mucosal mRNA expression of sGC-α1 and α2 subunits, both downregulated in gastric mucosa of rats exposed to I/R. We assume that CO-induced increase in GBF was dependent on NOS and partly mediated by sGC activity, both containing heme-binding domains [43,44].

Interestingly, mRNA expression for eNOS was not affected by I/R in gastric mucosa pretreated or not with CORM-2. However, mRNA expression of pro-inflammatory iNOS was upregulated within damaged tissue and pretreatment with CO donor decreased this mRNA fold change. Furthermore, exposure to I/R increased serum concentration of pro- and anti-inflammatory markers such as TGF-β2, TGF-β3, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, TNF-α, IFN-γ, GM-CSF and pretreatment with CORM-2 reversed this effect. Also, CO donor prevented I/R-induced DNA oxidation. Moreover, anti-inflammatory and anti-oxidative effect of CO has been reported previously [3]. Therefore, our results confirmed strong anti-inflammatory and anti-oxidative effect of CO released from CORM-2 as a part of the complex gastroprotective mechanism induced by this gaseous molecule.

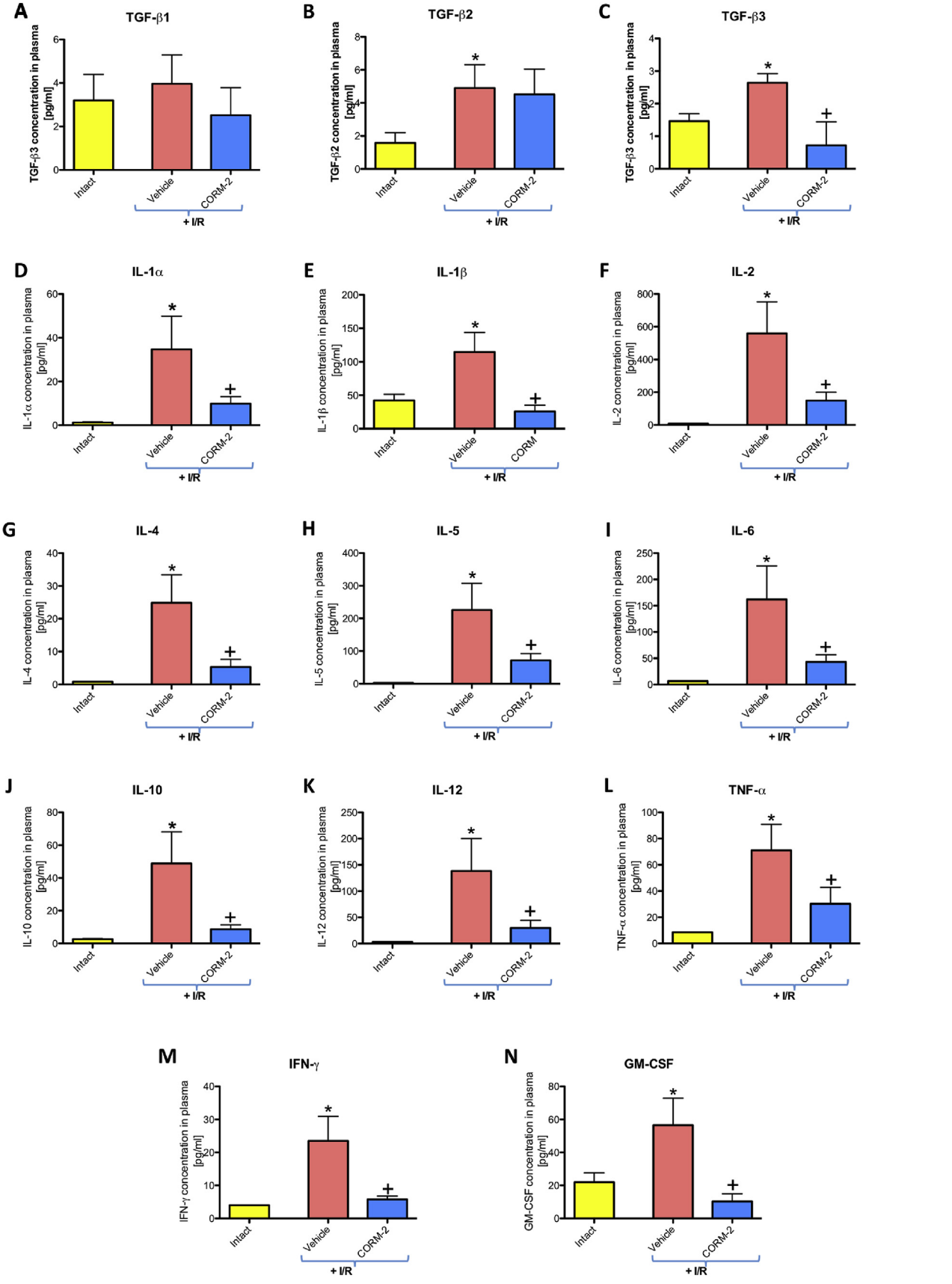

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Fig. 7. Concentration of transforming growth factor (TGF)-β1 (A), TGF-β2 (B), TGF-β3 (C), interleukin (IL)-1α (D), IL-1β (E), IL-2 (F), IL-4 (G), IL-5 (H), IL-6 (I), IL-10 (J), IL-12 (K), tumor necrosis factor (TNF)-α (L), interferon (IFN)-γ (M), granulocyte-macrophage colony-stimulating factor GM-CSF (N) in serum of rats pretreated with vehicle or tricarbonyldichlororuthenium(II) dimer (CORM-2, 5 mg/kg i.g.) and exposed to 3.5 h of ischemia/reperfusion (I/R). Intact refers to healthy gastric mucosa not exposed to I/R. Results are mean ± S.E.M of 5 samples per each experimental group. Asterisk indicates a significant change as compared with respective values obtained in intact gastric mucosa ($p < 0.05$). Cross indicates a significant change as compared with the values obtained in the vehicle-pretreated group exposed to I/R ($p < 0.05$).

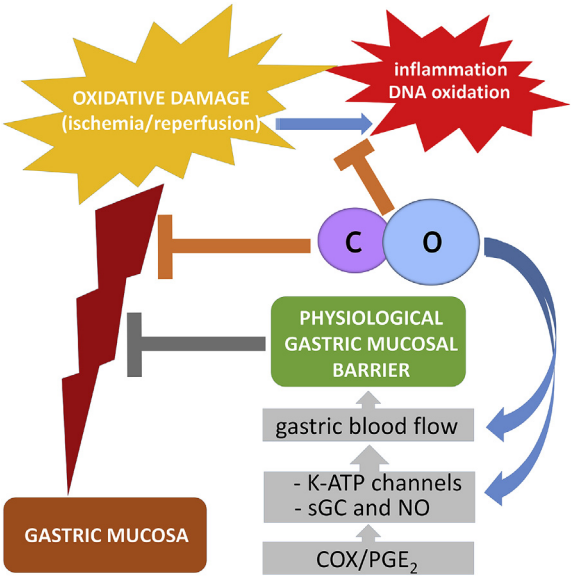

Fig. 8. Mechanisms involved in carbon monoxide (CO) mediated gastroprotection against ischemia/reperfusion-induced oxidative gastric damage. Footnotes: K-ATP channels- ATP-dependent potassium channels; sCG- soluble guanylyl cyclase; NO- nitric oxide; COX- cyclooxygenase; PGE_2- prostaglandin E_2.

5. Conclusions

Taken together, we conclude that the CO/HMOX-1/Nrf-2 patway is involved in the mechanism of I/R-induced gastric mucosal damage (Fig. 8). It is not excluded that the increase in mRNA expression for HMOX-1 ocurred possibly due to a positive defensive feedback mechanism in response to damaging effect of I/R. The pretreatment with CO releasing CORM-2 which increased both, the gastric mucosal CO content and COHb concentration resulting in enhancement in gastric microcirculation attenuated gastric lesions induced by I/R while decreased gastric DNA oxidation and inflammatory response at the systemic level as reflected by profound inhibition of proinflammatory cytokines. This CO-gastroprotection was mediated by the activity of sGC, NOS and K-ATP channels without affecting the mRNA expression of ATP-dependent potassium channels subunits Kir6.1 and Sur2 and soluble guanylyl cyclase (sGC)-α1 and sGC-α2 subunits. CO delivered from its donor maintained physiological gastric mucosal PGE_2 concentration but in contrast to our previous observation with CORM-2-induced gastroprotection against "contact" mucosal injury evoked by intragastric ethanol application which became reversible by indomethacin [30], the involvement of mRNA expression of either COX-1 or COX-2 in beneficial activity of this gaseous mediator at least in this model of microcirculatory perturbations, has not been observed.

Declaration of competing interest

None declared.

Acknowledgments

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