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Protective effect of carbon monoxide in transplantation

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Abstract

During the last decades due to the development of new immunosuppressive agents and improvements in organ preservation methods, surgical techniques, and postoperative care, organ transplantation has become an ultimate therapeutic option for irreversible organ failure. Early graft survival has significantly improved; however, the long-term outcome remains unsatisfactory. Multiple factors, both immunogenic and non-immunogenic etiologies, are involved in the deterioration of the allografts, and the recent use of expanded criteria donors to overcome the organ shortage may also contribute to the graft losses. Carbon monoxide (CO) is commonly viewed as a poison in high concentrations due to its ability to interfere with oxygen delivery. However, CO is endogenously produced in the body as a byproduct of heme degradation by the heme oxygenase (HO) and has recently received notable attention as a gaseous regulatory molecule. In fact, an augmentation of endogenous CO by induction of HO-1 or exogenously added CO is known to have potent cytoprotective effects in various disease models. Several recent reports have demonstrated that CO provides potent cytoprotective effects in the field of organ and cell transplantation. CO is able to prevent ischemia/reperfusion injury, allograft rejection, and xenograft rejection *via* its anti-inflammatory, anti-apoptotic and anti-proliferation effects, suggesting that CO might be a valuable therapeutic option in the field of transplantation. Based on the recent advancement of our understanding of CO as a new therapeutic molecule, this review attempts to summarize the functional roles as well as biological and molecular mechanisms of CO in transplantation and discusses potential CO application to the clinical transplant setting.

Keywords: carbon monoxide - transplantation - ischemia - reperfusion - rejection

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Introduction

Organ transplantation is an ultimate therapeutic option for irreversible organ failure. The introduction of new immunosuppressive agents, improvements in surgical techniques, and advances in post-transplant patient care have considerably improved early outcomes of organ transplantation. However, long-term patient outcomes have failed to yield comparable improvements. Long-term graft function and graft survival are affected by several factors, including early ischemia/reperfusion (I/R) injury, previous and/or ongoing alloimmune reactions, recipient metabolic abnormalities (elevated lipids or cholesterols), other recipient conditions (hypertension, viral infection), and adverse effects of chronic immunosuppressive treatment. Non-specific inflammatory events associated with these phenomena could trigger alloimmune reactions and promote deterioration of the grafts.

Furthermore, the current donor shortage has resulted in prolonged waiting times and increased deaths on the waiting list, and has led to the use of lower-quality organs. In 2005 in the United States alone, there were > 90,000 patients waiting for an organ transplant. Despite numerous efforts to increase the number of cadaveric organ donors to meet the growing demands, the number of cadaveric donors has remained relatively static (~ 6000 per year). This disparity results in thousands of deaths each year that might have been prevented if enough organs had been available. In an attempt to decrease the number of patient deaths while on the waiting list, the organ donor pool has been expanded with the use of marginal donors. This includes the use of older donors, non-heart beating donors, and grafts with prolonged cold storage. Organ grafts from these marginal donors have a higher incidence of severe cold I/R injury, resulting in an increased risk of delayed or primary graft non-function, enhanced alloimmune responses, developing other complications (*e.g.* infection), and more rapid graft deterioration. Thus, a number of concerns remain in clinical transplantation, and new strategies need to be developed to maintain the function of organ allografts and improve long-term outcomes of transplantation.

One of the promising strategies to protect the transplanted organs and cells from functional impairment is to utilize the heme oxygenase (HO), which is a rate-limiting enzyme converting heme into carbon monoxide (CO), iron, and biliverdin IXα [1] (Fig. 1). HO-1 is an inducible isoform of HO and has been shown to have potent protective effects against various stresses in diverse experimental models [2–5]. In the last decade, induction of HO-1 has been shown to be beneficial against pitfalls associated with organ transplantation, including I/R injury [6–9] and allogenic immune reactions due to histoincompatibility including graft rejection [4, 10–13] and graft-versus-host disease [14]. Likewise, carbon monoxide (CO), one of the byproducts of heme degradation through HO, has a wide range of effectiveness in preventing impairment of the transplanted grafts during I/R injury [15, 16], tissue injuries associated with acute rejection [17], and also mouse-to-rat xenograft rejection [18]. This review article summarizes the efforts made in understanding the cytoprotective effects of CO in organ transplantation in order to advance our knowledge of the potential clinical application of CO in the field of transplantation.

Heme oxygenase system

HO is the only enzyme by which a mammalian cell can catabolize heme into three byproducts: CO, iron, and biliverdin [1, 2]. Biliverdin is further converted to bilirubin through biliverdin reductase. To date, three isoforms of HO (HO-1, HO-2 and HO-3) have been identified [19–21]. HO-2 and HO-3 are constitutively expressed, whereas HO-1 is inducible. HO-2 is mostly concentrated in the brain and testis [22]. HO-3, more recently identified, is structurally similar to HO-2 but has lower enzymatic activity compared to other HO [21]. HO-1 is classified as a stress-inducible protein which is proven to be identical to heat shock protein 32 [23]. HO-1 is induced in response to various stimuli such as hypoxia, endotoxin, heat shock and reactive oxygen species (ROS) [24] and deliberated as an endogenous self-defense mechanism [25]. HO-1 is critical and indispensable to the survival of mammalians, and this paradigm is supported by observations of HO-1 deficient mice and humans. While HO-2 deficient mice can survive through their life span, HO-1 deficient mice frequently die in utero and only survive for less than one year, associated with marked splenomegaly and fibrosis [26, 27]. Similarly, an HO-1 deficient human patient is reported by Yachie

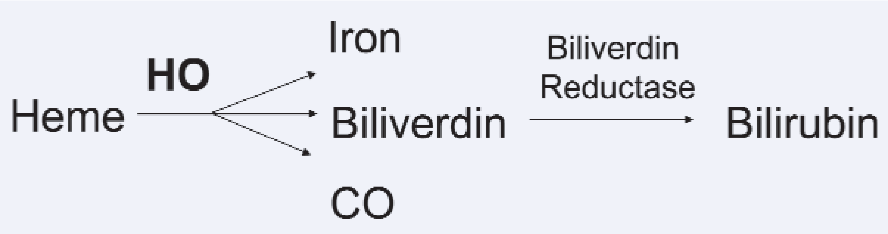

Fig. 1 Heme oxygenase system. Heme oxygenase (HO) is the enzyme which catabolizes heme into three byproducts: CO, iron, and biliverdin. Biliverdin is further converted to bilirubin through biliverdin reductase.

et al. [28]. The patient unfortunately died at the age of 6-years-old, and had demonstrated growth failure, anemia, iron deposition in the tissue, lymphadenopathy, leukocytosis, and susceptibility to oxidant injury. Thus, HO-1 certainly plays a crucial role in maintaining cellular homeostasis during various critical stress conditions.

In fact, the evidence suggests that HO-1 induction can be an efficient cytoprotective procedure in various disease models [12, 29–34]. In these models, administration of HO-1 inducers such as cobalt protoporphyrin (CoPP), hemin, and trinitrobenzene sulfonic acid or HO-1 gene transfer has been shown to provide beneficial effects, which are reversed by a specific HO inhibitor such as zinc protoporphyrin (ZnPP) or tin-protoporphyrin (SnPP). The mechanisms by which HO-1 can mediate these cytoprotective actions are not fully understood. However, three major catalytic byproducts of heme degradation, CO, biliverdin and free iron, are believed to be the effector molecules underlying the potent cytoprotection observed with the HO system.

Carbon monoxide (CO)

Endogenous CO in the body

CO, a diatomic gas, occurs in nature as a product of oxidation or combustion of organic matter. CO is an invisible, chemically inert, colorless and odorless gas that is commonly viewed as a poison in high concentrations. However, CO is endogenously produced in the body by enzymatic reaction as originally described by Tenhunen [1]; the catalytic breakdown of heme by constitutive HO system releases CO. Although CO is formally considered to be a catabolic waste product, HO-derived CO is the major source of intracellular CO, and a considerable amount of CO arises endogenously in mammalians. Although the values may vary depending on the environmental background, blood carboxyhemoglobin (COHb) is mostly generated from endogenous CO, and ranges between 0.4 and 3% [35, 36]. Increased levels of exhaled CO in the breath of humans are associated with several inflammatory or stress conditions [37, 38], and this can be explained by the increase of endogenous CO production by the elevation of HO-1.

Acute toxicity of CO

CO avidly binds to hemoglobin and forms COHb with an affinity 240 times higher than that of oxygen, possibly causing an interference with the oxygen-carrying capacity of the blood and consequent tissue hypoxia. Blood COHb levels well correlate to acute clinical symptoms. While COHb levels are ~ 3% in normal healthy adults, COHb can reach 10–15% in smokers [35, 36]. COHb levels of 10–30% can cause headache, shortness of breath, and dizziness, and higher levels (30–50%) produce deleterious toxicity, such as severe headache, vomiting, syncope, and arrhythmia [35]. Prolonged exposure to a toxic dose of CO becomes fatal at between 50 and 80% of COHb. Emergency treatment of CO poisoning to accelerate dissociation of CO from Hb, such as hyperbaric oxygen therapy, may be an aggressive supportive care to prevent death; however, > 10% of survivors are left with a presumed brain injury.

Chronic toxicity of CO

Chronic exposure to low concentrations of CO is not rare in the current living environment. As results from the increase of automobile exhaust air pollution, CO levels in major cities sometimes reach ~50 ppm and further increase to > 50 ppm in tunnels and garages. Occupational exposure to CO is also com-

Table 1 CO's protective effects in transplantation field

Author	Year	Journal	Organ	Animals	Combination	CO concentration	CO delivery	Reference
Ischemia/reperfusion								
Nakao	2006	Am J Transplant	intestine	rat	syngeneic	5%	soluble form	[57]
Kohmoto	2006	Surgery	lung	rat	syngeneic	250 ppm	inhalation	[98]
Kaizu	2005	Surgery	liver	rat	syngeneic	100 ppm	inhalation	[70]
Nakao	2005	Am J Transplant	heart, kidney	rat	syngeneic	20 ppm	inhalation	[84]
Seda-Neto	2004	Am J Physiol	kidney	rat	syngeneic	250 ppm	inhalation	[71]
Akamatsu	2004	FASEB	heart	rat	syngeneic	400 ppm	inhalation	[58]
Nakao	2003	Am J Pathol	intestine	rat	syngeneic	250 ppm	inhalation	[73]
Nakao	2003	GUT	intestine	rat	syngeneic	250 ppm	inhalation	[15]
Nakao	2003	Surgery	intestine	rat	syngeneic	250 ppm	inhalation	[16]
Clark	2003	Circ Res	heart	mice	syngeneic	CO-RM3	soluble form	[61]
Amersi	2002	Hepatology	liver	rat	syngeneic	300 ppm	inhalation	[75]
Allotransplantation								
Nakao	2006	Transplantation	heart	rat	fully allogeneic	20 ppm	inhalation	[79]
Sada-Neto	2006	Am J Physiol	kidney	rat	fully allogeneic	20 ppm	inhalation	[134]
Minamoto	2005	J Exp Med	airway	mice	fully allogeneic	250 ppm	inhalation	[136]
Martins	2005	Transplant Proc	kidney	rat	fully allogeneic	MC 100 mg/kg	orally	[63]
Song	2003	Am J Pathol	lung	rat	fully allogeneic	500 ppm	inhalation	[17]
Otterbein	2003	Nat Med	aorta	rat	fully allogeneic	250-1000 ppm	inhalation	[47]
Chauveau	2002	Am J Transplant	aorta	rat	fully allogeneic	MC 500 mg/kg	orally	[13]
Ke B	2002	Hum Gene Ther	liver	rat	fully allogeneic	MC 500 mg/kg	orally	[64]
Xenotranslantation								
Sato	2001	J Immunol	heart	mice to rat	mice to rat	250-400 ppm	inhalation	[18]
Islet transplantation								
Gunther	2002	Diabetes	islet	mice	syngeneic	1%	in culture	[145]
Wang	2005	Diabetes	islet	mice	fully allogeneic	250 ppm	inhalation	[59]

CO-RM: CO releasing molecule, MC; methylene chloride

mon to traffic polices, coal miners, and transportation mechanics, whose average COHb levels sometimes reach ~ 5% [35]. The National Institute for Occupational Safety and Health recommends an exposure limit for CO of 35 ppm for an 8-hour time-weighted average exposure, with a ceiling limit of 200 ppm for short-term exposure, which is designed to protect workers from health effects associated with COHb concentrations in excess of 5%. Chronic exposure to cigarette smoke also can produce elevated COHb levels to 2–10%, and heavy smokers can generate COHb levels as high as 15–18%. In addition, in some patients with blood diseases (*e.g.* hemolytic anemia), endogenous CO saturation reaches 6% [39, 40]. Although the influence of chronic CO exposure needs to be analyzed in detail, chronic exposures to low dose CO (< 50 ppm) are not likely to be fatal. Indeed, Stupfel shows that two years of continuous exposure of rodents to 50 ppm CO induces no significant alterations in physiological or biochemical parameters [41].

Cytoprotective actions of CO

CO, one of the byproducts of heme catalysis by HO, confers potent cytoprotection mimicking the protective effects of HO-1 overexpression, as mentioned above. A number of experimental studies have shown that CO at low levels (< 20% of COHb) has potent cytoprotective effects during oxidative stress, hyperoxic lung injury [42], endotoxemia [43], surgical ileus [44, 45] and arteriosclerosis [46]. The mechanisms underlying CO's cytoprotection are not fully understood. However, CO has been shown to exert biological actions by inhibiting pro-inflammatory cytokines and chemokines [47], preventing vascular constriction [48, 49], decreasing platelet aggregation and plasminogen activator inhibitor [50], and inhibiting apoptosis [51, 52]. Molecular and cellular mechanisms of these CO's renowned biological actions will be described below with a particular focus on organ transplantation (Table 1).

It should be mentioned that due to the affinity of CO for metal atoms, CO binds and forms complexes with numerous heme-containing proteins other than hemoglobin in the body, such as myoglobin, soluble guanylyl cyclase (sGC), inducible nitric oxide synthase (iNOS), cytochrome P450, cytochrome *c*, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. As a result of binding to the central iron group contained within these metalloproteins, CO potentially influences the biological activity of hemeproteins. It appears to depend on a signaling mechanism whether the binding may cause activations or inhibitions of these proteins. For instance, CO is shown to inhibit cytochrome P450 activity by the formation of complex between CO and cytochrome P450 [53]. Similarly, soluble CO inhibits NADPH oxidase cytochrome *b*558 activity [54]. On the other hand, the best characterized pathway of CO's actions is the activation of sGC, the enzyme that converts guanine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), which is an intracellular signaling molecule involved in the regulation of cellular events, such as smooth muscle relaxation, inhibition of platelet aggregation, and synaptic transmission. Further studies to understand CO's interaction to these hemeproteins, and possible alterations in their biological activity will provide useful information in developing therapeutic strategies using CO.

Delivery methods of CO

Since CO is a gaseous molecule, CO inhalation at a low concentration would be a straightforward delivery method to utilize CO as a therapeutic tool. CO-inhalation therapy could be a clinical strategy in transplantation because of its simple method of application during surgery and early post-transplant period. Adjustment of inhaled CO dose by blood COHb monitoring could also be helpful to avoid problems of CO-poisoning. However, as CO at high concentrations is known to be toxic, the secure and optimal delivery of gaseous CO needs to be carefully conducted. As mentioned above, a brief CO inhalation at a low concentration (~250 ppm) with COHb levels 10~20% does not induce significant morbidity in the previous small animals, large animals [45] and human studies [55].

Alternatively, CO can be used in soluble form by vigorously bubbling it into the solution. Using simple CO bubbling procedure, CO solubility in the solution can reach near theoretical equilibrium levels, calculated by Bunsen solubility coefficiency and Boil-Charles' law. For example, after lactate Ringer solution is bubbled for 5 min with 100% gaseous CO, the solution contains approximately 1000 µmol/L of CO, which is comparable to the theoretical solubility of CO at 20°C at 1 atm (1017.4 µmol/L). Intraperitoneal injection of 1.5 ml CO-saturated lactate Ringer into mice increases COHb levels to 8% at 5 min after injection [56], and COHb levels immediately return to the basal levels within 1 hr, indicating that CO in solutions could be an attractive CO delivery method to expand the application of CO. In organ transplantation, simple CO bubbling method could be used for the cold storage solution to ameliorate transplant-induced I/R injury. We have observed that cold storage of intestinal grafts in CO-bubbled UW solution ameliorates I/R injury following intestinal transplantation [57]. CO in preservation solution has been shown to protect heart grafts from I/R injury [58] and to improve islet graft viability [59].

CO-releasing molecules (CO-RM) are shown to exert a variety of pharmacological activities *via* the liberation of controlled amounts of CO into the biological systems. A wide range of CO carriers are currently available and include manganese (CORM-1), ruthenium (CORM-2 and -3), boron (CORM-A1) and iron (CORM-F3) [60]. In the transplantation field, CO-releasing molecules show protection of hearts [61] or kidney grafts against oxidative stress [62]; however, possible deposits of CO carrying heavy metals in the graft and/or recipient body remain a concern.

CO can be induced in the body by the administration of methylene chloride (MC), which is a prodrug metabolized almost exclusively in the liver and produces CO and carbon dioxide. When MC diluted in olive oil is given orally (500 mg/kg/d), COHb reaches the peak (5–10 %) at 2 hrs after MC administration, and the levels of COHb are maintained above the basal level for up to 24 hrs [13]. Chauveau *et al.* show that CO released from continuous MC treatment significantly suppresses intimal thickness due to chronic rejection of aortic allografts, more efficiently than HO-1 gene transfer with AdHO-1 [13]. Similarly, Martin *et al.* report that donor pretreatment with MC considerably reduces graft deterioration due to chronic allograft nephropathy in rats [63]. MC oral administration for 2 weeks prevents liver allograft rejection and suppresses hepatocyte necrosis [64]. Thus MC could be a useful experimental tool in delineating the CO's beneficial effects and mechanisms.

CO and ischemia/reperfusion (I/R) injury associating with transplantation

I/R injury in organ transplantation

Cold preservation is the current standard procedure to preserve cadaveric organs prior to transplants and involves intravascular flushing of the isolated organ using a hypothermic solution followed by storage at low temperature for the time required to transfer the graft to the recipients. Harvested organs are subjected to injury during cold preservation due to a hypoxic and hypothermic condition, and further damage is induced at reperfusion when warm oxygenated blood is reintroduced into the graft (warm reperfusion). Thus, organ transplantation procedure obligates cold preservation and warm reperfusion of grafts, resulting in some degree of cold I/R injury in all organ grafts. Multiple factors have been shown to contribute to the pathogenesis of I/R injury. The lack of oxygen during cold preservation induces depletion of ATP and shifts to anaerobic metabolism by the glycolysis pathway, followed by deterioration of the intracellular Ca^{++} and Na^{+} homeostasis and the activation of cytotoxic enzymes (*e.g.* proteases, phospholipases, endonucleases) [65, 66]. Subsequent warm reperfusion of grafts causes an excess of oxygen and generates ROS, which further promote cell damage [67]. As a result, damage or loss of vascular endothelial cells (VECs), disturbance of microcirculation, activation of potent inflammatory mediators (*e.g.* cytokines, adhesion molecules, platelet activating factors, eicosanoid products), and inflammatory infiltration are known to be characteristic features associated with I/R injuries [68, 69]. While the quality and quantity of tissue damage by I/R injury may vary among different types of organ grafts, the main component of this pathogenic process that is shared by all organs is the microvascular dysfunction (Fig. 2). The development of a treatment strategy that can prevent/ameliorate transplant-induced cold I/R injury will have a significant impact on patient care during the early post-transplant period, and potentially further increase the use of marginal organs.

VEC protection and vasorelaxation by CO

The main target of I/R injury is VECs. As mentioned above, the insult for VECs is initiated by hypoxic and hypothermic condition during cold preservation and amplified by warm reperfusion. Resulting VEC damage leads to a disturbance of graft microcirculation and subsequent activation of inflammatory cascade. The morphological changes of VECs caused by I/R injury can be seen

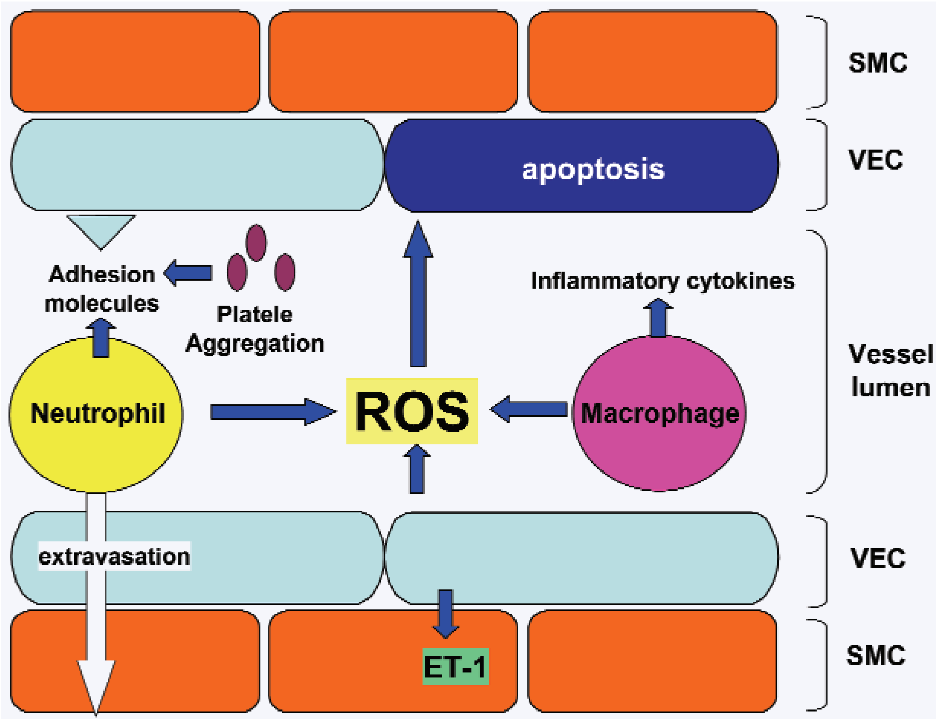

Fig. 2 Schema of I/R injury. Multiple events cause I/R injury associated with organ transplantation. A major target of I/R injury is VECs, leading to apoptosis, inflammation, production of ROS, and disruption of microcirculation.

in immunohistochemistry using monoclonal antibody specific for CD31 (pan endothelial cell marker, PE-CAM). In the intestine and kidney of the naïve unoperated animal, CD31 is abundantly expressed on VECs of the capillaries in the intestinal lamina propria and renal peritubular area. However, CD31 expression is considerably reduced, irregular, and interrupted during cold storage and reperfusion (Fig. 3). Similarly, scanning electron microscopy shows numerous vacuolization and disorganization of the internal cellular architecture of VECs, indicating the mitochondrial breakdown and irreversible degeneration in VECs induced by I/R [16, 70, 71] (Fig. 3).

CO is known to protect VECs *in vitro* culture system. Brouard *et al*. have shown that exogenous CO (10000 ppm) prevents TNFα-induced apoptosis of cultured VECs [51]. Zhang *et al.* have also shown CO's protective effects on rat primary pulmonary VECs against anoxia-reoxygenation injury [72]. These CO's protective actions are mediated *via* the activation of p38 mitogen-activated protein kinase (MAPK), since SB203580, selective p38 MAPK inhibitor abrogates CO's beneficial effects [51, 72].

CO's protective effects for VECs are also seen in *in vivo* I/R injury animal models after transplantation. We have reported that renal and intestinal graft VECs in CO-treated recipients are well-preserved morphologically with vital linear CD31 stain and intact intracellular architectures in transmission electron microscopy. Further, three-dimensional visualization of the fine vascular arrangements in the transplanted grafts during I/R injury with scanning electron microscopy reveals exudation and significant numbers of filling defects in the capillary networks in the control intestine or kidney grafts. However, inhaled CO (250 ppm) provides almost complete protection of vascular integrity, showing fine features of vascular network as in normal organs [71, 73] (Fig. 3).

VEC injury due to I/R also causes upregulation of adhesion molecules (*e.g.* ICAM-1, VCAM-1, P-selectin) to promote VEC-leukocyte and -platelet interactions and leads to leukocyte extravasation, disturbance of blood circulation, and graft tissue injuries. We have shown that CO inhalation decreases ICAM-1 mRNA expression in the grafts after reperfusion [71, 73]. Morisaki *et al.* have shown that endotoxin-treatment of rats augments rolling and adhesion of leukocytes in the mesenteric venules, which is inhibited by CO [74]. CO also has a number of other crucial functions on VECs to regulate coagulation and platelet aggregation, which will be discussed in the following section.

In addition to the direct effect of CO on VECs, CO acts as a vasorelaxing mediator *in vivo*.

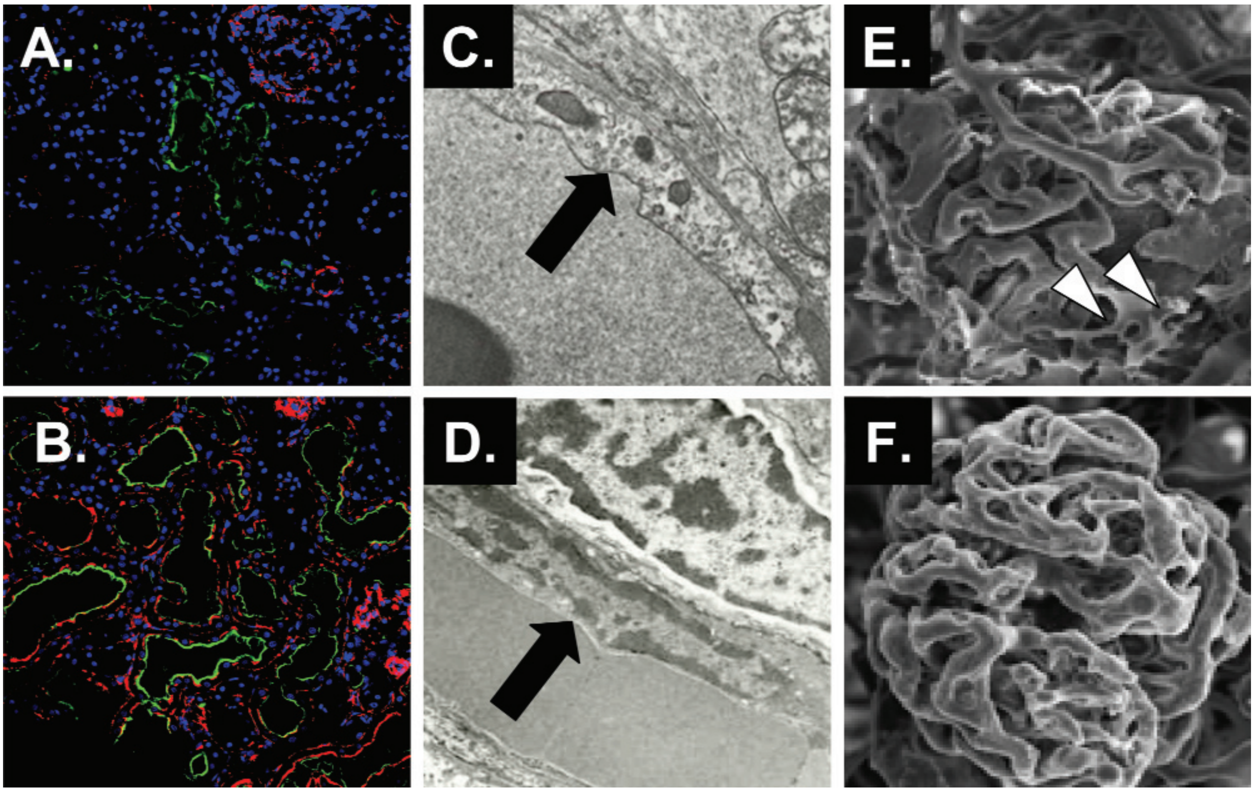

Fig. 3 CO's protective effects for vasculatures. Morphological changes of VECs caused by I/R injury and amelioration with CO treatment were shown. Immunohistochemistry using CD31 (green) in conjunction with rhodamine-phalloidin (red) stain for the kidney graft 1 hr after reperfusion revealed considerably reduced, irregular, and interrupted CD31 stain in air-control grafts (**A**, x 400), indicating severe VEC injury. CO-treated grafts demonstrated relatively normal CD31 expression (**B**, x 400), suggesting the preservation of VECs with CO during cold I/R stress. Transmission electron microscopy for small intestinal grafts taken 1 hr after reperfusion demonstrated injured VECs in the lamina propria, showing karyolitic nuclei with disorganized internal architecture (**C**, x 5000). Endothelial cells in the CO-treated grafts were well maintained and demonstrated a normal intracellular architecture (**D**, x 5000) Three-dimensional visualization of the fine microvascular architecture of the renal glomerular tuft revealed renal I/R injury in air-control grafts with an exudation and pooling of casting resin, vasoconstriction and a significant number of filling defects in the capillary network, indicating interruptions in vascular continuity (**E**, x 900, arrow head). Kidney grafts with inhaled CO showed the preserved glomerular tuft architecture, with protection against capillary leakage, microcircular interruption, and vasoconstriction (**F**, x 900).

Although multiple factors may be involved in regulating vascular tone, smooth muscle cells (SMCs) surrounding vessels play key roles in the arteries. As SMCs directly communicate with VECs through a hole in the internal elastic lamina, the interaction between VECs and SMCs is believed to be important in the regulation of vascular resistance. I/R injury significantly impairs graft microcirculation, and this is reflected by a marked elevation of vascular perfusion pressure after cold preservation [62] or reperfusion [75]. During I/R injury, damaged VECs release a variety of vasoconstructive proteins such as endothelin (ET)-1, which is the most powerful natural mammalian vasoconstrictive agent [76]. For instance, an elevation of ET-1 has been reported in renal I/R injury [77], and the blockade of ET_A receptor prevents I/R injury in rat cardiac transplantation model [78]. CO inhalation at 250 ppm prevents ET-1 activation after cardiac transplantation and improves graft outcome [79]. Stanford *et al.* have reported that CO can inhibit ET-1 release from human pulmonary artery VECs and SMCs [80], supporting CO's beneficial effects on ET-1 suppression and inhibition of vasoconstriction. Additionally, CO dilates vessels in hypoxic condition by directly activating calcium-dependent potassium channels in the small arteries and relaxing SMCs [81].

Furthermore, CO plays a key role in regulating vasomotor tone through the activation of sGC and subsequent generation of cGMP. An increase in cGMP leads to the activation of cGMP effector proteins, such as cGMP-dependent protein kinases (PKG), cGMP-regulated phosphodiesterases (PDEs) and cGMP-activated ion channels. Activation of PKG is mandatory for cGMP-mediated relaxation of vascular SMCs and inhibition of platelet aggregation [82]. *In vitro* experiments clearly show that exposure of vascular SMCs to exogenous CO elevates intracellular cGMP concentrations, which may explain CO's vasorelaxation mechanism [83]. Our laboratory has demonstrated that recipient CO inhalation treatment at 250 ppm significantly improves graft blood flow determined by Doppler flow meter [71, 73, 84]. This beneficial effect is completely reversed by a selective sGC inhibitor, 1H-[1, 2, 4] oxadiazolo [4, 3-a] quinoxalin-1-one (ODQ), suggesting that the CO's vasodilative effects are mainly mediated by sGC/cGMP pathway.

Similar to other organs, pre- and post-sinusoidal vascular resistance in the liver is presumably controlled by the vascular SMCs at the terminal portal venules and hepatic venules. However, the hepatic sinusoids are mostly regulated by stellate cells and sinusoidal endothelial cells through the vasoactive mediators including ETs and angiotensin II (vasoconstricting agents), and nitric oxide and CO (vasorelaxing agents). Stellate cells lining the outer surface of sinusoidal walls constitute a well-organized meshwork of intercellular connection and exhibit contractile phenotypes like vascular SMCs in response to a variety of vasoactive substances. Stellate cells also possess abundant activity of sGC which may serve as a cellular target for gaseous mediators such as CO [85]. Using an isolated perfused rat liver model under constant flow conditions, Suematsu *et al.* show that metalloporphyrin, an HO inhibitor, diminishes endogenous CO levels in the effluent and increases perfusion pressure. These effects are reversed by exogenous CO or cGMP analogues in the perfusate [86]. Amersi *et al.* analyze portal venous resistance after 24 hrs cold storage of rat liver grafts using *ex vivo* perfusion circuit and show that perfusion with blood devoid of CO results in a significant increase of vascular resistance and that the supplementation of 300 ppm CO into the perfusate decreases portal venous resistance. These effects are sGC-independent, but mediated by activation of p38 MAPK [75]. Multiple factors appear to be involved in CO's protection of graft vasculature. Detailed mechanisms and interactions of each contributing factor need to be studied further; however, considering that the injury to the graft vascular system is the fundamental cause of transplant-induced I/R injury, diverse layers of protection afforded by CO would be particularly valuable.

CO acts as an anti-coagulation factor

Platelets are known to play an important role in the pathogenesis of I/R injury. Thromboxane A2 and prostaglandin I2 are major prostanoids mainly released from VECs and platelets. I/R injury increases the release of thromboxane A2, a potent platelet activator, and on the contrary, decreases the release of prostaglandin I2, a potent platelet anti-aggregatory factor [87, 88]. Under these circumstances, platelet activation, aggregation, and adhesion to the endothelium may occur immediately after reperfusion, resulting in microvascular thrombosis and accumulation of fibrin. CO is known to inhibit the coagulation cascade and possess a potent anti-coagulation property. Brune *et al.* report that CO can inhibit thromboxane release from the platelets *in vitro* [89]. Fujita *et al.* demonstrate in lung warm I/R injury model that CO inhalation reduces deposition of fibrin in the microvasculature with a suppression of plasminogen activator inhibitor (PAI)-1 [50]. PAI-1 inhibits the activity of tissue-type plasminogen activator (t-PA) and reduces fibrinolysis. The beneficial effects of CO in this study, as well as the suppression of PAI-1, are abolished by the inhibition of sGC activity, suggesting that these actions are mediated by sGC/cGMP pathway. Morisaki *et al.* have clearly demonstrate that CO attenuates sevoflurane-induced microvascular endothelial interactions with leukocytes and platelets using *in vivo* rat mesenteric venules [90]. The same group also shows that CO attenuates endotoxin-induced platelet aggregation and leukocyte adhesion through glycoprotein Ibα mediated mechanism, while endotoxemia causes a marked depression of platelet velocity accompanied by augmented rolling and adhesion of leukocytes in venules [74]. Presumably, CO's anticoagulation effects also contribute to the protection of I/R-induced VEC damage.

CO has an anti-inflammatory effect

Although transplant-induced I/R injury is initiated during cold storage, warm reperfusion triggers the inflammatory process by the activation of chemical mediators and enzymes (*e.g.* ROS, phospholipase A2, lysozymes, leukotrienes, prostaglandins). Significant cellular damage also activates macrophages and other graft parenchymal cells and results in a release of numerous inflammatory mediators, including TNFα, IL-1 and IL-6, followed by an extravasation of macrophages, neutrophils and T cells to the graft interstitial space [91]. In addition, VEC injury causes the upregulation of adhesion molecules (*e.g.* ICAM, VCAM) and strengthens the adhesion between the leukocytes and VECs, promoting extravasation of leukocytes out of the circulation [92]. As a result, neutrophils infiltration plays an important role in the late inflammatory responses and enhances graft parenchyma damage by secreting proteolytic enzymes such as elastases [93], generating ROS, and physically impairing the microcirculation. Thus, the inflammatory events in the early phase of I/R is generally mediated by graft cells, while late injury is induced by recipient circulating neutrophils cells [94].

CO has been known to exert anti-inflammatory actions in various injury models. Typically, LPS-induced inflammatory tissue injury is inhibited by CO with a downregulation of pro-inflammatory cytokines (*e.g.* TNFα, IL-1β, IL-6) [47, 95–97]. Since transplant-induced I/R injury involves vigorous inflammatory reactions, the use of CO to ameliorate I/R injury in the transplant setting is a straightforward application. In the series of rodent experiments in our laboratory, recipient CO exposure at a dose of 250 ppm for 1 hr before and 24 hrs after transplantation significantly reduces inflammatory cell infiltration in I/R injury induced in small intestine, kidney, heart, lung and liver grafts with extended cold preservation and following transplantation [15, 16, 70, 71, 98]. The levels of mRNA for pro-inflammatory mediators including IL-6, TNFα, IL-1β, inducible nitric oxide (iNOS) and cyclooxygenase (COX)-2 rapidly elevate peaking 1 to 3 hrs after reperfusion after transplantation. CO inhalation significantly decreases the elevation of these mediators, associated with a decrease of cellular infiltration in the grafts (Fig. 4).

These inflammatory processes in I/R injury are regulated through multiple intracellular signaling cascades involving numerous transcription factors. Nuclear factor κB (NFκB) is an important member of inflammatory transcription factors and has been known to play a pivotal role in I/R injury associating with various organ transplantations. During the I/R injury process, nuclear translocation and DNA binding of NFκB is promptly initiated with the phosphorylation and degradation of IκB-α, an inhibitory protein of NFκB activation [99–101]. In the model of hepatic I/R injury after 18 hrs UW cold preservation, prompt NFκB activation is observed within 1 hr after graft reperfusion. Interestingly, CO 100 ppm inhalation does not inhibit NFκB DNA binding activity or IκB-α phosphorylation [70]. The study suggests that although CO consistently downregulates I/R-induced pro-inflammatory cytokine activation, protection against cold I/R injury-induced inflammatory responses by inhaled CO is independent of NFκB signaling cascade. However, the involvement of NFκB in anti-inflammatory effects of CO has been an arguable topic in studies outside of transplantation. While anti-inflammatory effects of inhaled CO in the ventilator-induced lung injury in rats are shown to be independent of NFκB pathways [102], inhibition of LPS-induced GM-CSF production in cultured macrophages with a low concentration of CO is mediated *via* the inhibition of NFκB activation and IκB-α phosphorylation [43].

Other important signaling pathways in I/R injury are the MAPKs, which are signal transduction enzymes that integrate cellular responses to the environmental stresses [103]. In addition to participating in many normal host functions, the MAPKs have been implicated in inflammatory diseases [104]. The pathways include parallel and interacting cascades that phosphorylate the three main MAPK families, p38 MAPK, extracellular signal-regulated kinase (ERK), and c-Jun NH2 terminal protein kinase (JNK). MAPKs are the most widespread mechanisms of eukaryotic cell regulation, and each of MAPK pathways is preferentially recruited by distinct sets of stimuli through diverse receptor families, thereby allowing the cells to respond coordinately to multiple divergent inputs.

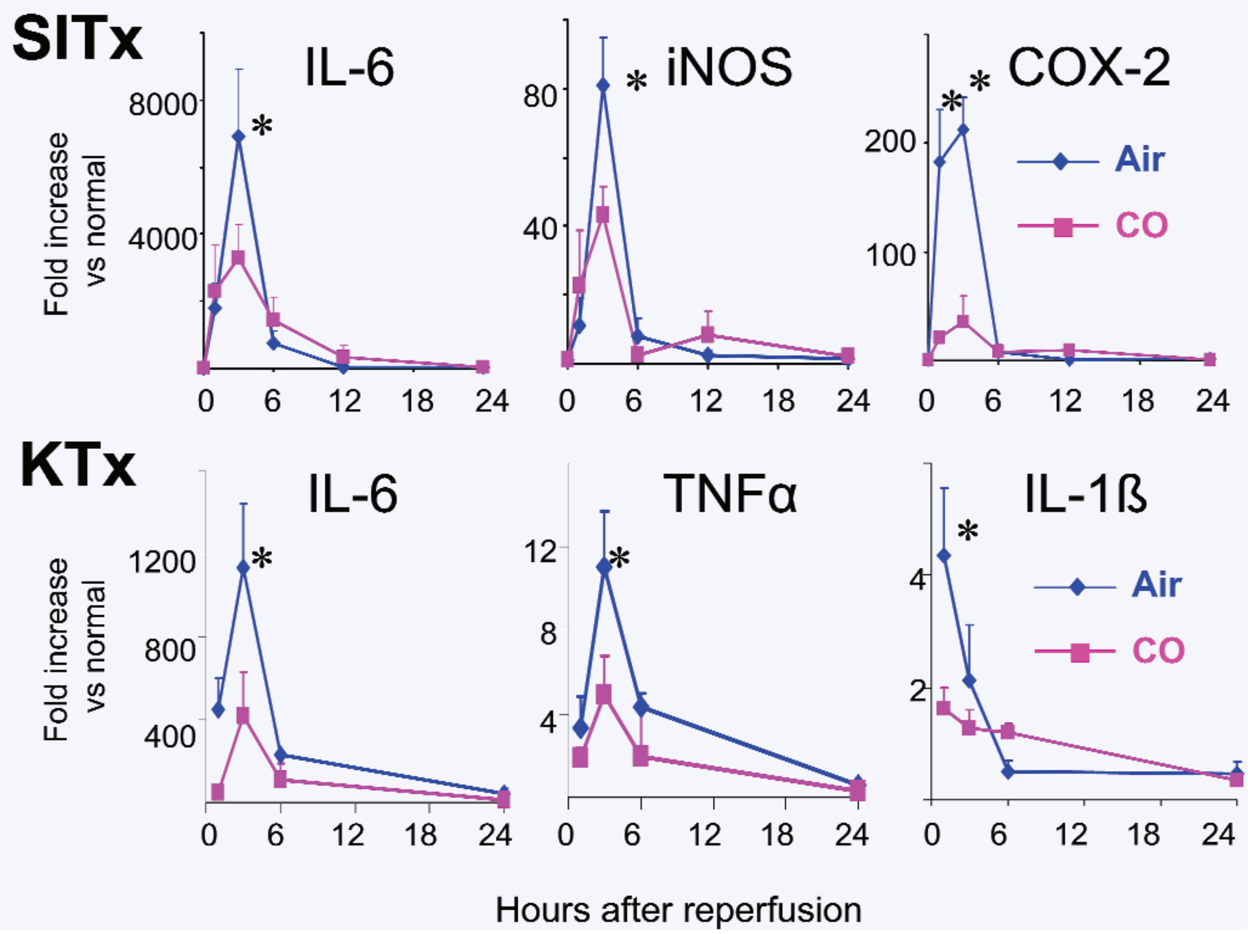

Fig. 4 CO's effects on mRNA levels of inflammatory mediators after I/R injury. Inhibition of I/R injury-induced mRNA upregulation for inflammatory mediators with exogenously inhaled CO (250 ppm) after small intestinal transplantation (SITx) and kidney transplantation (KTx). (* $p < 0.05$ *vs.* CO)

Anti-inflammatory properties of CO have been shown to be mediated *via* the regulation of MAPKs; CO inhibits LPS-induced pro-inflammatory cytokine production (*e.g.* TNFα, IL-1β) while simultaneously increasing expression of anti-inflammatory cytokine IL-10 in mice and RAW 264.7 macrophages *via* p38 MAPK activation [47]. CO protects cultured human alveolar epithelial cells from hyperoxic damage by the selective activation of p38 MAPK and its upstream MAPK kinase (MKK) kinases [105]. TNFα-stimulated rat pulmonary artery VECs show phosphorylation of ERK1/2, JNK1/2, and p38 MAPK, and CO (1%) exhibits marked attenuation of the phosphorylation of ERK1/2 and upstream MEK 1/2 kinase and accentuation of phosphorylated p38 MAPK [106]. These studies emphasize a potential mechanism of CO modulating the inflammatory response by differentially activating the MAPKs.

In transplant experiments, although the absolute significance of MAPKs and its up-stream signals or down-stream transcription factors in promoting or inhibiting I/R injury has not been adequately addressed, accumulated data indicate that I/R injury activates all 3 major MAPK pathways [107–109]. Interestingly, while numerous studies examining the efficacy of CO in experimental inflammatory responses have shown the association of CO's anti-inflammatory function with the activation of MAPKs, we have observed significant inhibition of ERK1/2 with CO in I/R injury models associating with transplantation. Lung grafts preserved for 6 hrs in UW demonstrate significant I/R injury with an activation of 3 major MAPKs after transplantation into syngeneic rats. CO inhalation (250 ppm) improves lung graft function, increases PaO_2, and downregulates pro-inflammatory cytokines. Beneficial effects of CO inhalation in this study associate with nearly complete inhibition of ERK1/2 activation [98]. In *ex vivo* rat liver perfusion circuit model, CO's anti-inflammatory effects, such as a decrease of cell infiltration, are abrogated by a p38 MAPK inhibitor, SB 203580 [75].

In addition, the inhibition of MAPKs during transplant-induced I/R injury is shown to be protective; selective JNK inhibitor CC-401 reduces hepatic injury and increases recipient survival in 40 hrs hepatic I/R injury model without affecting p38 and ERK1/2 activation [110]. FR167653, a p38 MAPK inhibitor also demonstrates the protection against rat hepatic I/R injury and canine cardiac I/R injury [111–113]. Production of TNFα and impaired myocardial function in human atrial trabeculae exposed to 45 min hypoxia are inhibited with a p38 MAPK inhibitor, SB 203580 [114]. Thus, these studies demonstrate that the inhibition of prompt MAPK activation during I/R injury is beneficial and suggest the possible therapeutic approach to inhibit MAPK pathways. CO's anti-inflammatory action might be mediated through the MAPKs; however, actual roles of the MAPKs in inflammatory responses of I/R injury during CO treatment will require further investigation.

CO inhibits apoptosis

I/R injury of organ grafts involves both necrosis and apoptosis; however, the relative contribution of each pathway has been a debate [69, 115]. After reperfusion of cold preserved organ grafts, tissue necrosis is a common pathophysiological finding due largely to adjacent tissue inflammation. Although apoptosis of graft parenchymal cells is documented in I/R injury, it is unclear whether apoptosis is the primary event associated with I/R injury in organ transplantation or if it is induced secondarily following the inflammatory response of I/R injury. Thus, the role of apoptosis during I/R injury needs to be studied further; however, inhibition of the cell death pathway has been shown to be an important strategy for the prevention of I/R injury [116, 117], suggesting that apoptosis could be equally important as necrosis in the development of graft injury and dysfunction following I/R injury. Interestingly, in spite of the general view that apoptosis and inflammation/necrosis are two largely independent processes, apoptotic cell death has been shown to be a crucial event in initiating inflammation and subsequent tissue injury [118].

CO is known to have anti-apoptosis effects in both *in vivo* and *in vitro* models. CO prevents TNFα-induced apoptosis of cultured VECs [51] and fibroblasts [119] mainly *via* the activation of p38 MAPK. The anti-apoptotic effect of CO in VECs is also shown to depend on the activation of signal transducer and activator of transcription (STAT)3 *via* phosphatidylinositol 3-kinase/Akt and p38 MAPK pathways with a subsequent attenuation of Fas expression and caspase 3 activity [120]. Liu *et al*. demonstrate that activation of sGC by CO results in the inhibition of apoptosis of vascular SMCs by blocking the release of the mitochondrial cytochrome *c*, essential for apoptosis induction, and by inhibiting expression of the proapoptotic protein p53 [52]. Thus, a direct inhibition of apoptosis afforded by CO may be mediated by several different mechanisms, probably depending on the variety of stimuli to induce apoptosis and the types of responding cells.

In intestinal transplantation, intestinal crypts are extremely vulnerable to oxidative stress due to the large proliferative demands and high mitochondrial respiration. In immunohistochemical analysis, activated caspase-3 positive cells increase in 6 hr UW cold preserved intestinal grafts at 1 hr after reperfusion in syngeneic rat recipients, with an upregulation of mRNA for pro-apoptotic gene Bax. Inhaled CO (250 ppm) in recipient animals significantly reduces caspase-3 positive crypt epithelial cells with a downregulation of early Bax mRNA and upregulation of anti-apoptotic Bcl-2 [16]. In renal I/R injury induced by 24 hrs cold preservation and transplantation in the rat, activated caspase-3 is expressed on isolated tubular epithelial cells and cells/debris in the tubular lumen. The number of positive cells rapidly increases and peaks at 6 hrs after reperfusion in control grafts. Recipient CO inhalation (250 ppm) reduces tubular cell apoptosis, and the peak number of caspase-3 positive cells is decreased to $< 50\%$ [71]. Akamatsu *et al.* have reported that when CO is administered to the donor and to the recipient (400 ppm) as well as during 24 hrs cold storage in 1% CO-saturated UW solution, heart graft survival in syngeneic rat recipients

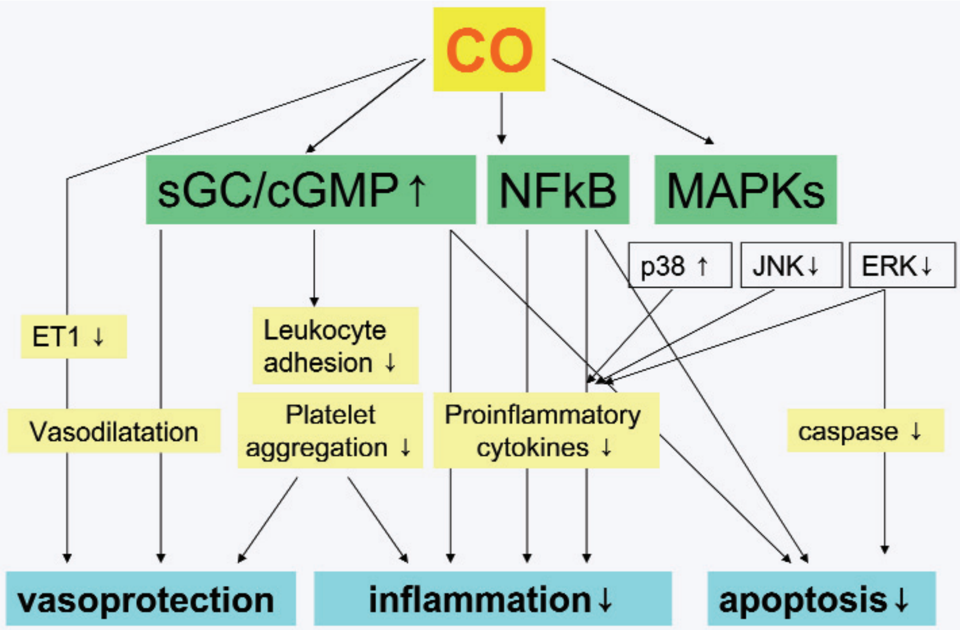

Fig. 5 Summary of the mechanism of CO's protection for I/R injury.

increases to 83% from 0% in untreated controls. Beneficial effects in this study are associated with the decrease of TUNEL positive apoptotic cells in the myocardium at 10 min after reperfusion [58].

Although the biological function of CO in ameliorating I/R injury associating with organ transplantation appears to be substantiated, it needs to be determined if CO acts in a protective manner primarily by preventing I/R injury-induced apoptosis of graft cells. Numerous experimental studies, in particular *in vitro* experiments, suggest a potent anti-apoptotic action of CO, and further studies investigating roles of apoptosis in I/R injury, CO's anti-apoptotic action in I/R injury, and involved signaling mechanisms will be particularly productive.

Enhanced protection with combination of another byproduct, biliverdin

Biliverdin or bilirubin, another byproduct of heme catalysis, has been shown to provide protection against oxidative stress by scavenging peroxyl radicals. Bilirubin is formed from biliverdin by biliverdin reductase and oxidated to biliverdin with ROS. This catalytic anti-oxidant cycle between bilirubin and biliverdin could amplify the anti-oxidant effect of biliverdin or bilirubin alone [121], and is considered as the best anti-oxidant system against lipid peroxidation [122–124]. We and others have shown that biliverdin-bilirubin significantly reduces I/R injury [125–128]. We demonstrate that the combination therapy of CO and biliverdin provides enhanced protection against I/R injury in the rat heart and kidney transplantation models [84]. Considering that CO and biliverdin/bilirubin might inhibit I/R injury *via* the different mechanisms, combination therapy would be an attractive strategy. Multiple mechanisms of CO's protection against transplant I/R injury are summarized in Fig. 5.

CO and allograft rejection

Pathophysiology of allograft rejection

Generally, three main types of rejection may occur: hyperacute, acute, and chronic in clinical transplantation. Hyperacute rejection, occurring within minutes to days of transplantation, is due to preformed IgG antibodies in the recipient that react against HLA antigens in the transplanted organ. Acute rejection occurs most frequently in the first 6 months after transplantation and is mainly mediated by T cells, which infiltrate the allograft, undergo clonal expansion, and cause tissue destruction. Immunosuppressive drugs are most effective in preventing this type of rejection. Chronic rejection is the term used when allograft function slowly deteriorates and there is histologic evidence of arteritis obliterance (AO) and fibrosis. For all organs, the pathophysiology of chronic rejection is similar: progressive intimal hypertrophy of the small to medium-sized arteries, interstitial fibrosis and atrophy, and eventual failure of the organ transplant. In addition, organ-specific features are also known as chronic allograft nephropathy in kidney grafts and bronchiolitis obliterant (BO) in lung allografts. Although chronic rejection is most likely to occur later in the post-transplantation course, it may develop as early as 6 to 12 months after transplantation. Unfortunately, there is no standard treatment for chronic rejection. Virtually all patients with chronic rejection suffer serious side effects of chronic immunosuppression. Although the underlying mechanisms that lead to this form of injury remain poorly understood, it is generally considered to be multifactorial, involving both alloantigen-dependent and alloantigen independent non-immunologic factors.

CO prevents T cell proliferation

Allograft rejection is mediated primarily by T cells, with B cells playing a role *via* antibody production. Cellular allograft rejection involves recipient T cell recognition of HLA molecules expressed on donor-derived antigen-presenting cells (direct allorecognition) or presentation of donor-derived peptides by recipient antigen-presenting cells to recipient T cells (indirect allorecognition). Once the alloantigens are recognized, the activation, proliferation, and production of cytokines by T cells and other immune cells lead to the amplification of the alloimmune response. This complex process involves the generation of effector T cells, antibody production by activated B cells, and macrophage activation. Several *in vitro* studies have shown that CO is capable of directly inhibiting T cell proliferation and IL-2 secretion without affecting cell viability. Anti-T cell proliferative effect of CO is mediated *via* the selective inhibition of ERK pathway in human $CD4^+$ T cells [129]. Using p21 knockout mouse T cells, CO is also shown to inhibit T cell proliferation *via* the expression of $p21^{cip1}$ and inhibition of caspase activity [130]. These studies stress the active role of CO in immune responses, and CO might directly downregulate alloreactive immune responses.

CO prevents allograft rejection

Since CO is shown to directly regulate *in vitro* T cell proliferation, acute allograft rejection could be ameliorated with CO treatment. In addition, as CO is known to prevent inflammation and apoptosis associated with I/R injury, we can expect that CO may reduce inflammatory responses involved in allograft rejection. Although we are not able to observe heart allograft survival prolongation with inhaled CO (20 ppm) in the fully allogenic rat transplant model without immunosuppression [79], Song and Kubo *et al.* have demonstrated that CO inhalation (500 ppm) alone considerably ameliorates acute lung allograft rejection using the rat orthotopic lung transplantation model [17]. The lung allografts treated with CO manifest less cellular infiltration, less intravascular coagulation and less intraalveolar hemorrhage. Transplanted lungs of CO-exposed recipients also display decreased apoptotic alveolar cell death compared with the untreated transplanted lungs, as assessed by TUNEL and caspase-3 immunostaining. CO exposure inhibits the induction of pro-inflammatory genes, including macrophage inflammatory protein (MIP)-1α and macrophage migration inhibitory factor (MIF), and growth factors such as platelet-derived growth factor, all of which are upregulated in untreated allografts. Similarly, Ke *et al*. show CO's anti-rejection effects using fully allogenic rat liver transplantation model [64]. In their study, liver recipient treatment with MC for 2 weeks significantly enhances recipient survival to a median of 21 days, while all untreated rats die within 10 days after liver transplantation. Although it needs to be determined if CO directly regulates T cell proliferation *in vivo* transplant setting, CO's beneficial effects in these studies appear to include anti-inflammation and anti-apoptosis, similar to those seen in previous reports of I/R injury models as described above.

CO ameliorates allograft vasculopathy and other chronic changes

Chronic rejection is pathologically characterized by the development of AO associated with arterial intimal thickening. Intimal thickening is caused by the migration of SMCs into the intima from the media and/or proliferation of resident or migratory SMCs and elaboration of extracellular matrix. Leukocytes recruited in response to injury or inflammation also populate the thickened intima in graft arteriosclerosis [131]. Several *in vitro* experiments have demonstrated CO's anti-proliferation effects on vascular SMCs. CO is shown to be produced by vascular SMCs under hypoxic conditions, and inhibiting CO penetration or scavenging CO with hemoglobin increases vascular SMC proliferation in response to serum or mitogens (*e.g.* endothelin), whereas increasing CO production or exposing cells to exogenous CO leads to a markedly attenuated growth response [132]. Peyton *et al.* also show that exogenous CO inhibits serum-stimulated vascular SMC proliferation *via* cell cycle arrest at G1/S transition phase and selective blockade of expression of cyclin A without affecting the expression of cyclin D1 and E [133]. Otterbein *et al.* show that CO's anti-proliferative effects on mice vascular SMCs require $p21^{Cip1}$ expression, as well as sGC and p38 MAPK activation [46].

In rats, *in vivo* intimal thickening at 14 days after balloon angioplasty of the carotid artery is effectively inhibited with 1 hr pretreatment of 250 ppm CO inhalation. Subsequently, CO (250 to 1000 ppm) inhalation applied in the rat aortic transplantation model has been shown to reduce intimal thickening and cellular infiltration [46]. Chauveau *et al.* report that oral MC administration for 30 days (d0-30) significantly reduces vascular SMC infiltration into the intima and suppresses intimal thickness in the rat aortic grafting model [13]. We have shown that 20 ppm of CO inhalation for 4 weeks significantly suppresses graft arteritis in heart allografts at 50 days. Notably, daily 1 hr exposure to CO 250 ppm, instead of continuous 20 ppm inhalation, for 28 days (d0–28) significantly promotes graft survival, suggesting that brief exposure to CO, a more practical CO inhalation protocol, is sufficient to exert a therapeutic effect [79].

In our model of fully allogenic kidney transplantation under brief tacrolimus immunosuppression, continuous recipient exposure to low dose CO (20 ppm) for 4 weeks also minimizes chronic allograft nephropathy. Renal allograft function in air controls progressively deteriorates, and creatinine clearance declines to less than 10% of naïve animals by 80 days with substantial proteinuria. CO-treated animals show significantly better creatinine clearance with minimal proteinuria [134]. Additionally, CO exposure prevents the disease progression and supports functional recovery of kidney allografts, even after chronic allograft nephropathy is established. The data suggests that CO decelerates chronic deterioration of the grafts and reverses established chronic allograft nephropathy [135].

Likewise, the pronounced involvement of airway, bronchiolitis obliterant (BO), is a clinically devastating feature in chronic lung allograft rejection. The histological feature of BO is characterized by peri-bronchiolar leukocyte infiltration, associated with a later abnormal epithelial-mesenchymal repair response and fibro-proliferation. This leads to luminal obliteration of respiratory bronchioles by the deposition of collagen matrix. In the rat nonvascularized tracheal transplantation model, significant luminal narrowing was observed in allografts at 3 weeks after transplantation. CO exposure to the recipient at 250 ppm for 2 weeks significantly reduces graft luminal occlusion [136]. *In vitro* cell culture experiments have demonstrated that CO plays an important role in modulating human airway SMC proliferation *via* inhibition of cyclin D1 expression or G0/G1 arrest mediated by G1-cyclin-dependent protein kinase inhibitor $p21^{cip1}$ [137]. CO acts as an anti-proliferation signal on SMCs by inhibiting NADPH oxidase, which is known to generate ROS as signaling intermediates and promote airway SMC proliferation [138]. It would be interesting to explore if these effects of CO against SMC proliferation would be sufficient to suppress BO development during pulmonary chronic rejection process in the lung transplant model.

CO prevents fibrosis

Although various factors are involved in chronic rejection development, the final common pathway is the failure in proper repairing and excessive repair, subsequent replacement of the original tissues by fibrotic tissue, and consequent loss of organ function. Tissue repair is initiated by inflammatory and fibrogenetic signaling followed by interstitial mononuclear and macrophage infiltration, and tissue remodeling involves a variable extent of fibroblast proliferation and deposit of extracellular matrix. Ongoing interstitial fibrosis is also known to associate with microvascular injury and loss of interstitial capillaries, followed by tissue hypoxia [139].

Given the known anti-proliferative effects of CO as described above, it is postured that the mechanism of anti-fibrosis, at least in part, might be through the inhibition of fibroblast proliferation with CO. Zhou *et al.* have reported that CO suppresses *in vitro* proliferation of cultured fibroblast with increased cellular levels of $p21^{Cip1}$ and decreased levels of cyclins A and D. This effect is independent of the observed suppression of MAPK's phosphorylation by CO but dependent on increased cGMP levels. The same effects are seen in *in vivo* mice bleomycin-induced lung fibrosis model, and suppression of collagen-1 production and matrix deposition by fibroblast depend on the transcriptional regulator Id1 [140].

In the process of chronic allograft nephropathy, chemokine-chemokine receptor interaction may play a significant role in mononuclear cell recruitment, leading to interstitial fibrosis [141, 142]. We have shown that the expression of chemokine MCP-1, regulated upon activation normal T-cell expressed and secreted (RANTES), and CCR1 increases gradually in the renal allografts by 80 days. CO inhalation at 20 ppm for the first 4 weeks significantly inhibits the activation of these chemokines, resulting in reducing fibrosis and cellular infiltration. CO inhalation at 20 ppm also reduces collagen type I and IV, as well as TGF-β expression, in the kidney allografts [134]. Taken together, CO's anti-fibrotic effects may involve its anti-proliferation and anti-inflammation actions.

CO and xenotransplantation

Due to the current problem of shortage of organs in clinical transplantation, xenotransplantation, interspecies transplantation of organs, is frequently suggested as alternative approach to overcome allograft shortage. As a source of organs, pigs are currently considered to be the most suitable. However, major problems remain to be resolved before successful clinical transplantation can be initiated; the most challenging of these problems is immunological. Mouse to rat transplantation is a widely used concordant xenotransplantation model to study xenogenic antibody-mediated immune reactions. Using this model, Sato *et al.* have demonstrated the CO's role in prolonging cardiac xenograft survival [18]. Prolonged mouse cardiac graft survival in rat recipients with transient complement depletion by cobra venom factor and cyclosporine A immunosuppression is abrogated when HO-1 activity is inhibited by the specific HO inhibitor, SnPP. Heart xenografts are rejected within 7 days with microvessel platelet sequestration, thrombosis of coronary arterioles, IgM and C1q vascular deposition, myocardial infarction and apoptosis of VECs as well as cardiac myocytes. Under the inhibition of HO-1 activity with SnPP, exogenous CO (250–400 ppm) suppresses graft rejection and restored long-term graft survival to more than 50 days. Under CO exposure, there are no signs of vascular thrombosis as revealed by the lack of detectable P-selectin-expressing platelet aggregates or intravascular fibrin. This observation strongly supports that the protective effect of HO-1 in terms of preventing xenograft rejection is mediated *via* CO.

CO and pancreatic islet transplantation

Type I diabetes mellitus is an autoimmune disease that causes the destruction of pancreatic β cells. The patients suffering from type I diabetes are obliged to be completely insulin dependent. Transplantation of human islets of Langerhans is becoming an established procedure for treatment of type I diabetes [143]. Islet transplantation can provide physiologic insulin control with euglycemia and correction of HbA1c. However, the long-term outcome for transplanted islets has not been promising, although the number of cases increases since the release of the Edmonton Protocol [144]. To prevent primary nonfunction of islets, maintenance of islet viability would be critical. Specially, prevention of apoptosis that occurs soon after islet transplantation would improve function of the transplanted islets and reduce the number of islets needed to treat diabetes.

Based on the previous reports, we can prospect that CO may be applicable to maintain islet viability. Gunther *et al*. demonstrate that islets cultured in the presence of 1% CO are protected from TNFα-induced apoptosis [145]. In this study, the islets cultured in the presence of CO show significantly better-function after transplantation into syngeneic diabetic mice, compared to those without CO. Inhibition of sGC with ODQ suppresses anti-apoptotic effects of CO, suggesting that sGC is an important mediator of CO's actions in this model. Same group has shown that CO inhalation treatment for donor and recipient prolongs DBA/2 mouse islet allograft survival in streptozotocin-induced diabetic B6AF1 mice [59]. Surprisingly, CO inhalation only to the donor for 20 hrs before isolation also leads to long-term survival of transplanted islets in untreated allogeneic recipients. Although macrophages and T cells are likely to be involved in the initial immune reaction against the pancreatic islets, CO treatment leads to less infiltration of recipient macrophages into the transplanted islets. Thus, CO's multiple protective mechanisms including anti-apoptosis and anti-inflammation might be involved in the islet protection.

Future scope and conclusion

Studies accumulated over the last several decades have demonstrated that CO provides potent cytoprotection in a wide variety of *in vitro* and *in vivo* injurious models. These studies have set the premise that CO could be useful in protecting organ grafts from various injurious insults associated with transplantation and encouraged us to strive for additional scopes of CO to the field of organ transplantation. Ongoing investigations in experimental transplantation studies have revealed that CO confers tissue protection during I/R injury, alloimmune responses, and chronic allograft deterioration due to fibroinflammatory reactions. A key challenge in the future would be to translate these current exciting discoveries into new therapeutic modalities in the clinical transplantation setting. Unique clinical circumstances of transplantation allow multiple therapeutic strategies to apply CO to the cadaveric donor, excised grafts, and transplanted recipients. This exceptional situation could be beneficial in developing safe and effective CO delivery methods of CO. A more comprehensive understanding of the toxicity, pharmacokinetics, and biology of CO will certainly assist to harness the protective potential of CO.

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