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Hypergravity induces expression of cyclooxygenase-2 in the heart vessels

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Abstract

Cyclooxygenase-2 (COX-2), a rate-limiting enzyme for prostaglandin biosynthesis, is induced by various stimuli including mechanical stress, and plays important roles in pathophysiological conditions. For example, gravitational stress has been shown to induce expression of COX-2 in bone tissues, which is essential for bone homeostasis. To investigate whether COX-2 is induced by gravitational loading in other tissues than bone, we exposed mice to hypergravity at 2G and 3G for 4 hours. We demonstrate here that COX-2 is induced in the mouse heart vessels by hypergravity. Moreover, hypoxia-inducible factor (HIF)-1α and its downstream genes such as inducible nitric oxide synthase (iNOS), vascular endothelial growth factor (VEGF) and heme oxygenase (HO)-1 were induced in the heart simultaneously, while none of these genes were induced in the COX-2 (–/–) mouse heart. Therefore, COX-2 induced in the heart helps protect the heart function against hypoxia under hypergravity condition through HIF-1α induction.

Keywords: COX-2, hypergravity, heart, hypoxia, HIF-1α, iNOS, VEGF, HO-1

Introduction

Astronauts experience hypergravity of 3.2G at launch and 1.4G on reentry, and aviators of jet fighters are exposed to accelerated hypergravity. To prevent unwanted outcomes by exposure to such an increased gravity, it is helpful to understand the biological responses to hypergravity using animal models. It has been well investigated that mechanical stress caused by the gravity is important to maintain the bone mass, because a reduced gravity decreases the mass and mechanical properties of human and animal bones [1]. Prostaglandins (PGs) have been shown to mediate the gravity-dependent bone formation and maintenance of bone structure [2]. Moreover, fluid shear stress by cultured medium on osteoblasts induces expression of cyclooxygenase (COX)-2, the rate-limiting enzyme for PG biosynthesis [3]. Furthermore, the serum-dependent COX-2 induction in osteoblasts is suppressed significantly when cells are placed under microgravity in a space shuttle [4]. These results demonstrate that the gravity-dependent COX-2 induction is required for the bone tissue homeostasis. However, it has not been investigated whether a gravitational loading induces COX-2 in other tissues than bone.

Under the normal gravity, COX-2 is induced essentially in inflammatory lesions and tumor tissues, which plays a key role in the respective pathological conditions [5-7]. Although cytokines and tumor promoters mediate COX-2 induction in these lesions, direct mechanical stress also induces COX-2 in normal tissues. For example, increased inner pressure in the bladder and glomeruli induce COX-2 expression in the bladder detrusor

muscle and glomerular podocytes, respectively [8, 9]. Moreover, fluid shear stress and increased luminal pressure of vessels induce expression of COX-2 in the vessel walls [10-12]. Accordingly, it is possible that mechanical stimuli caused by gravitational force stimulate COX-2 expression in other tissues than bone, and affect their physiological functions.

To assess this possibility, we examined the mRNA levels for COX-2 in various tissues after exposing mice to hypergravity at 2G or 3G for 4 hours. We demonstrate here that hypergravity induces COX-2 expression in the lung, brain and heart. In the heart, expression of hypoxia-inducible factor (HIF)-1α and its downstream genes were upregulated by hypergravity in a COX-2-dependent manner. It has been reported recently that inhibition of COX-2 in the heart causes increased risks of myocardial infarction and stroke [13-16]. Moreover, COX-2 is induced by mild ischemia in the heart, and plays a protective role against ischemic stress [17, 18]. Accordingly, our results suggest that the hypergravity-dependent COX-2 expression in the heart has cardioprotective effects against possible ischemic stress under hypergravity.

Materials and methods

Animals Female COX-2 (–/–) mice (n=3) and wild-type littermates (n=6) at 20 weeks of age were used for each hypergravity experiment at 2G, 3G and control 1G. Generation of COX-2 gene knockout mice was described previously [6]. Animal experiments were carried out with ethical committee approvals at Kyoto University (Kyoto, Japan) and Japan Aerospace Exploration Agency (JAXA, Tsukuba, Japan).

Centrifugation Experiments Mice were subjected to hypergravity for 4 hours using Centrifugal Acceleration Test Facility at JAXA. Centrifugation with a 7.25 m-arm at 15.70 rpm and 19.23 rpm produced 2G and 3G hypergravity conditions, respectively. A cage-mounting module was attached at the end of the arm that allowed one degree freedom, thereby ensuring that the net G field was perpendicular to the cage floor. Temperature and moisture in the cages were maintained at 22.5 ^{0}C and 42 ± 2%, respectively.

Reverse-Transcription Polymerase Chain Reaction (RT-PCR) Mice were euthanized immediately after centrifugation, and tissue samples were collected in RNAlater solution (Qiagen). Total RNA was extracted from the colon, small intestine, stomach, kidney, lung, brain, and heart using ISOGEN solution (Nippon Gene, Tokyo, Japan). To determine the expression levels of genes, extracted RNA was reverse-transcribed and amplified with PCR using specific primers. The genes analyzed were for COX-1, COX-2, HIF-1α, inducible nitric oxide synthase (iNOS), vascular endothelial growth factor (VEGF), and heme oxygenase (HO)-1. The following primer sets were used for the respective genes: COX-1

(F-5'-GAGATGCGCCTACAGCCCTT-3', and R-5'-GCGTCTCACTAAGACAGACC-3'); COX-2 (F-5'-CAAACTCAAGTTTGACCCAG-3', and R-5'-GCCGGGATCCTTTTACAGCTCAGTTGAACG-3'); mPGES-1 (F-5'-CCGAATTCTTGAAGTCCAGGCCGGCTAG-3', R-5'-TAATGTCGACACCAAGTCCGCAAGTTC-3'); HIF-1α (F-5'-CATACAGTGGCACTCACAGTC-3', and R-5'-CGTAACTGGTCAGCTGTGGT-3'); iNOS (F-5'-TGTCAGTGGCTTCCAGCTCC-3', and R-5'-TAGTCTTCCACCTGCTCCTC-3'); VEGF (F-5'-GCCAAGTGGTCCCAGGCTGC-3', and R-5'-CTGTGCTGTAGGAAGCTCAT-3'); and HO-1 (F-5'-TGAGCTGTTTGAGGAGCTGCA-3', and R-5'-GAAACGGATATCAAAGTGGCC-3'). Specific GAPDH primers were used for the internal control to normalize the sample cDNA amounts. Band intensities of the RT-PCR products were quantified using Image J application (NIH). Statistical analyses were carried out by Student's *T*-test, and *p*-values < 0.05 were considered as significant.

Immunohistochemistry Localization of COX-1 and COX-2 in the heart was investigated by immunohistochemistry. The heart tissues were fixed in 4% paraformaldehyde, embedded in paraffin wax, and sectioned at 4-μm thickness. Sections were microwave-treated to retrieve antigens, blocked with 10% goat serum, and incubated with the primary antibodies. Rabbit polyclonal antibodies for COX-1 (Santa Cruz Biotechnology) and COX-2 (Cayman Chemical) were used at 100- and 500-fold dilutions,

respectively. Staining signals were visualized using Vectorstain Elite Kit (Vector) according to the manufacturer's protocol. Serial sections were stained with hematoxylin and eosin (H&E) and examined histologically.

Results and Discussion

We first examined expression of COX-2 under normal gravity (1G) by RT-PCR in the colon, small intestine, stomach, kidney, lung, brain and heart (Fig. 1*A*). The COX-2 mRNA was detected in most tissues examined, which is consistent with a previous report [19]. However, COX-2 expression was detected only weakly in the heart. When mice were placed under the 3G condition for 4 hours, the COX-2 mRNA level was increased significantly in the lung, brain and heart, although it remained unchanged in other tissues (Fig. 1*A*). We then quantified band intensities of the RT-PCR products from the lung, brain and heart of both 2G- and 3G-exposed mice, and determined the relative mRNA levels to that of the 1G-control (Fig. 1*B*). In the lung and brain, the COX-2 mRNA levels were increased approximately 1.5 times both at 2G and 3G. In the heart, however, COX-2 expression was markedly elevated to 2.5 and 2.8 times in the 2G- and 3G-exposed mice, respectively. It is likely that hypergravity at 2G is strong enough for the maximum COX-2 induction in these tissues, because the COX-2 level at 3G was almost the same as that at 2G. While the direct metabolite of arachidonic acid by COX-2 is PGH_2, it is further converted to PGE_2 by an inducible enzyme mPGES-1 that appears to be functionally coupled with COX-2 [20]. Moreover, we have demonstrated recently that simultaneous expression of COX-2 and mPGES-1 *in vivo* results in elevated tissue PGE_2 levels [21]. Interestingly, the levels of mPGES-1 mRNA in the heart and brain were significantly higher under hypergravity than those at 1G (Fig. 1*C*). Accordingly, it is likely that hypergravity enhances PGE_2 production

in the heart and brain through simultaneous induction of both COX-2 and mPGES-1.

Accumulating evidence indicates that COX-2 plays a protective role in the heart from infarction and stroke (ref. 20 and *see below*). Accordingly, we examined heart samples for further analyses. First, we determined COX-2-expressing cells in the heart by immunohistochemistry. In the 3G-exposed mouse heart, a strong immunostaining signal for COX-2 was found in the vessel wall (Fig. 2*B*), while it was not detected in the 1G-control mice (Fig. 2*A*). Abundant COX-2 staining in the 3G-exposed mouse heart was found both in the vascular smooth muscle cells and endothelial cells (Fig. 2*B*, inset). We did not find any histopathological lesions in the heart of the 3G-exposed mice, when compared with that of the control mice (Fig. 2*C* and *D*). It has been reported that not only COX-2, but also COX-1 is induced in the vessel walls, either by the increased fluid shear stress or increased intraluminal pressure [11, 12], although COX-1 is generally expressed constitutively. To examine the possibility whether shear stress or increased luminal pressure of the heart vessels was involved in the hypergravity-dependent COX-2 induction, we determined expression of COX-1 in the heart by immunohistochemistry. Notably, a stronger signal for COX-1 was detected in the vessel wall of the 3G-exposed mouse heart than in the 1G-control heart vessel (Fig. 2*E* and *F*). Consistent with the immunostaining results, the COX-1 mRNA level determined by RT-PCR was increased 1.9- and 1.8-fold in the 2G- and 3G-exposed mouse heart, respectively (Fig. 3). Induction of both COX enzymes in the vessel walls strongly suggest that shear stress or increased inner pressure of

the vessels stimulates COX-2 expression [11, 12]. It is possible that hemodynamic changes under hypergravity increased blood volume in the heart vessels, causing shear stress and increased pressure. Importantly, induction of COX-1 in the heart was independent of COX-2 expression, because the same induction of COX-1 was found in the COX-2 (–/–) mouse heart under 2G and 3G conditions (Fig. 3).

Recently, it has been reported that users of high-dose rofecoxib (Vioxx), a COX-2 selective inhibitor, were 1.7 times more likely than non-users to have coronary heart disease [13], and that long term use of rofecoxib increased risk of myocardial infarction and stroke [15, 16]. One of the possible reasons for the heart problems by COX-2 inhibition is thrombosis by suppressing COX-2-dependent PGI_2 synthesis in the endothelial cells [10, 22]. PGI_2 potently inhibits aggregation of platelets through vasodilation and preventing the proliferation of vascular smooth muscle cells. On the other hand, it has been demonstrated that COX-2 expression and subsequent PGE_2 production are induced in the heart during ischemic preconditioning which is an adaptive response of the heart to a mild ischemic stress and plays a key role for cardioprotection against myocardial infarction [17, 18, 23]. To examine whether hypergravity-induced COX-2 expression contributes to cardioprotection from ischemic stress, we analyzed expression of HIF-1α in the hypergravity-exposed mouse heart (Fig. 3). It has been shown that COX-2 induces HIF-1α at both transcriptional and post-transcriptional levels [24, 25]. Interestingly, HIF-1α expression was elevated significantly in the heart both at 2G and 3G. Importantly, such induction of HIF-1α was not

found in the COX-2 (–/–) mouse heart under the same conditions, indicating that COX-2 is responsible for HIF-1α induction in the heart under hypergravity. We thus determined expression of HIF-1α target genes, vascular endothelial growth factor (VEGF), inducible nitric oxide synthase (iNOS) and heme oxygenase (HO)-1, which may protect heart tissues from ischemia by stimulating angiogenesis and production of vasodilators [26]. Notably, expression was increased significantly for iNOS, VEGF and HO-1 in the 2G- and 3G-exposed mouse heart, compared with that of the 1G-control (Fig. 3). Furthermore, none of these genes was upregulated in the COX-2 (–/–) mouse heart (Fig. 3). These results, taken together, indicate that hypergravity stimulates COX-2 expression in the heart vessels, which is responsible for further induction of HIF-1α and downstream iNOS, VEGF and HO-1. It is of great interest to investigate whether these hypoxia-inducible factors are also induced in the preconditioned heart in a COX-2-dependent manner [17, 18, 23]. Notably, COX-1 expression did not compensate for induction of these genes under hypergravity. Because mPGES-1 is functionally coupled with COX-2, induction of COX-1 may result in production of other prostanoid than PGE_2. It has been demonstrated that COX-1 (–/–) mice show increased cardiac ischemic/reperfusion injury (21). Therefore, it is possible that COX-1 is involved in the ischemic preconditioning through a PGE_2/HIF-1α-independent pathway.

Although short term ischemic stress caused by coronary occlusion induces COX-2 in the rabbit and mouse heart [18, 23], such ischemic stress is unlikely under the 2G or 3G hypergravity. Consistently, hypoxic signs such as necrosis were never found in the 3G-

exposed mouse heart (Fig. 2*D*). These results rule out the possiblity that COX-2 is induced by ischemic stress under hypergravity. Rather, it is likely that blood stream in the coronary vessels is increased under hypergravity, causing the COX-2 induction described above.

Importantly, we did not find any histopathological lesions in the lung, brain or heart of COX-2 (–/–) mice (data not shown). It is possible that hypergravity *per se* is not the direct cause of tissue hypoxia, but such hypergravitational responses rather help prevent myocardial infarction and stroke from possible subsequent ischemic stresses. Astronauts are exposed to hypergravity at launch and aviators of jet fighters also experience sustained gravitational forces. Under such circumstances, aviators' blood is pushed downwards, and it is therefore possible that oxygen supply to the heart muscle falls less than the demand. Induction of COX-2 can play a protective role in such situations. Therefore, our results caution the possibility that use of the COX-2 inhibitors and non-steroidal anti-inflammatory drugs under hypergravity increase the risk for myocardial infarctions and strokes.

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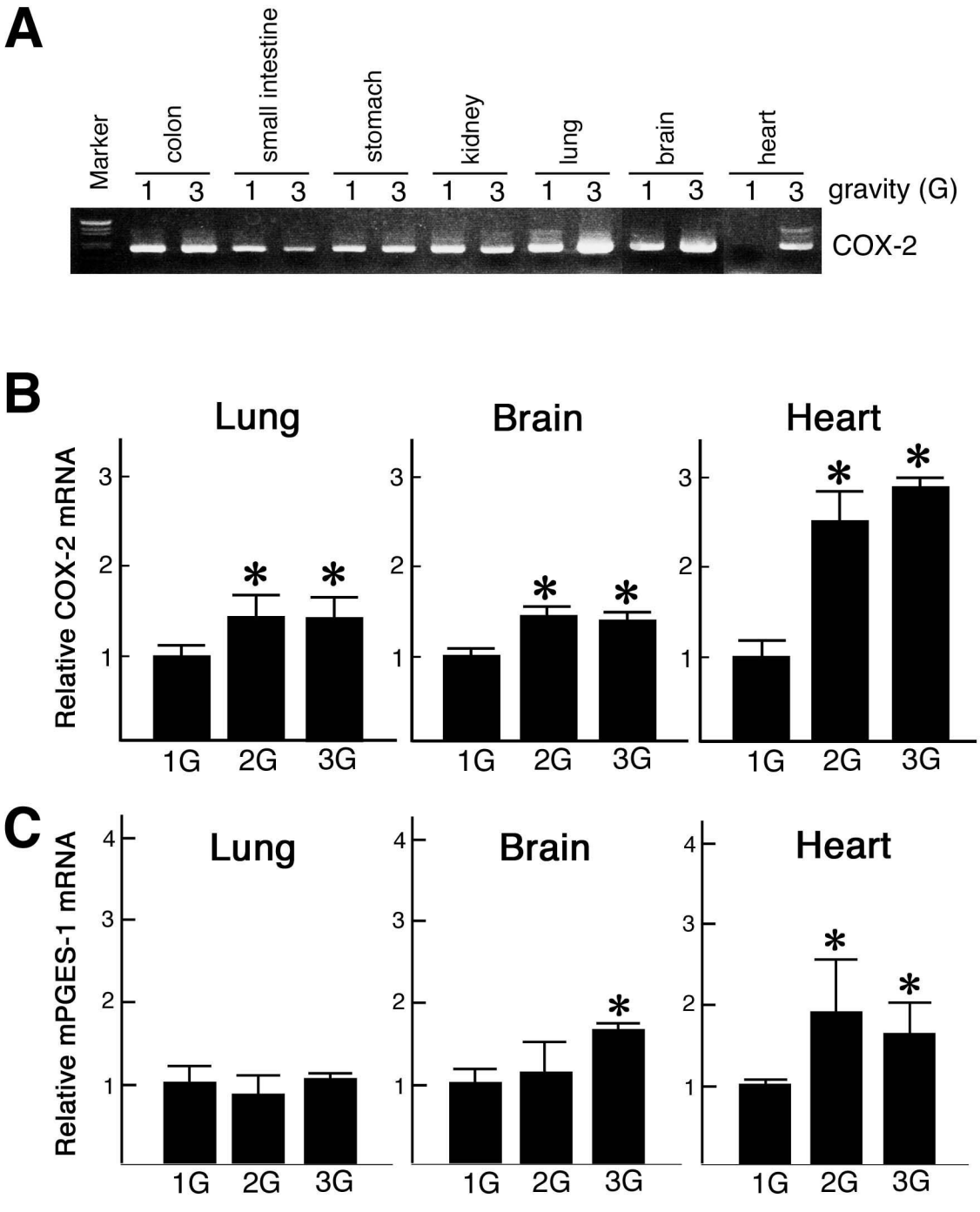
Figure Legends

Fig. 1. Expression of COX-2 at indicated gravitational conditions. (A) Representative RT-PCR at 1G control (-) and 3G (+) in the colon, small intestine, stomach, kidney, lung, brain and heart. (B, C) Relative mRNA levels of COX-2 (*B*) and mPGES-1 (*C*) in the lung, brain and heart at 2G and 3G, compared with that at 1G are presented as the mean ± s.d. *, $p <$ 0.05 to 1G control.

Fig. 2. Immunohistochemistry of COX-2 in the heart under 1G control (*A*) and 3G (*B*). Inset in (*B*) shows the heart vessels at a higher magnification. Near by serial sections of (*A*) and (*B*) are shown in (*C*) and (*D*), respectively (H&E). Immunostaining for COX-1 in the heart at 1G (*E*) and 3G (*F*) are indicated. Asterisks indicate vessels. Arrowheads in (*B*) and in (*F*) show positive staining for COX-2 and COX-1, respectively. Arrows in the inset (*B*) point to endothelial cells and smooth muscle cells that express COX-2. Expression of COX-2 is not detected in the 1G-control heart vessels (*A*), whereas weak COX-1 staining is found in the control vessel (*E*). Bars indicate 200 μm.

Fig. 3. Expression of COX-1, HIF-1α, iNOS, VEGF and HO-1 at 2G and 3G compared with that at 1G in the heart of the COX-2 wild-type mice (open bars) and COX-2 (–/–) mice (black bars). The relative mRNA levels are presented as the mean ± s.d. *, $p <$ 0.05 to 1G control of the respective COX-2 genotypes.

Figure 1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Brain
Heart
Relative COX-2 mRNA
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C
Relative mPGES-1 mRNA
1G 2G 3G

Figure 2

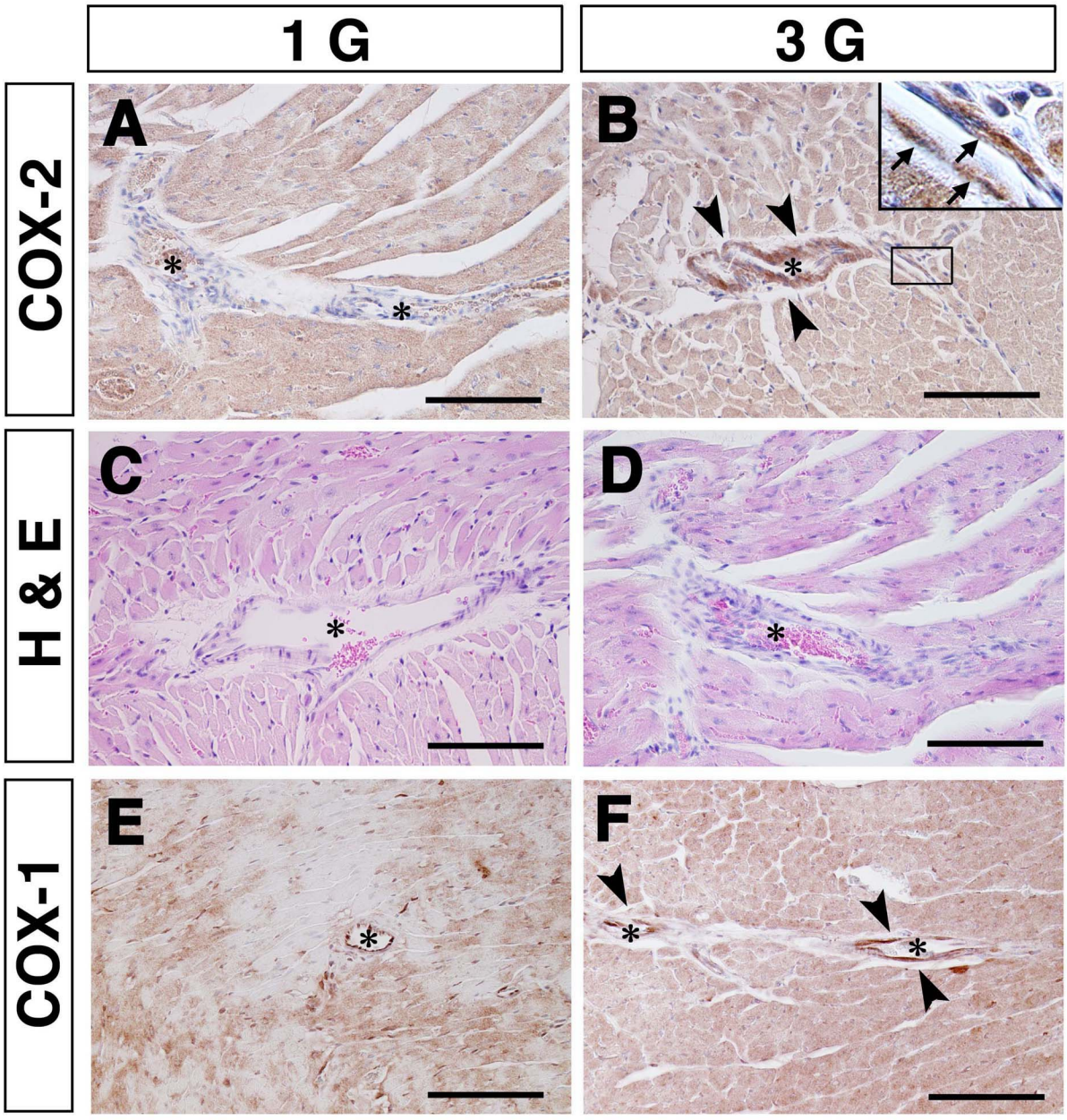
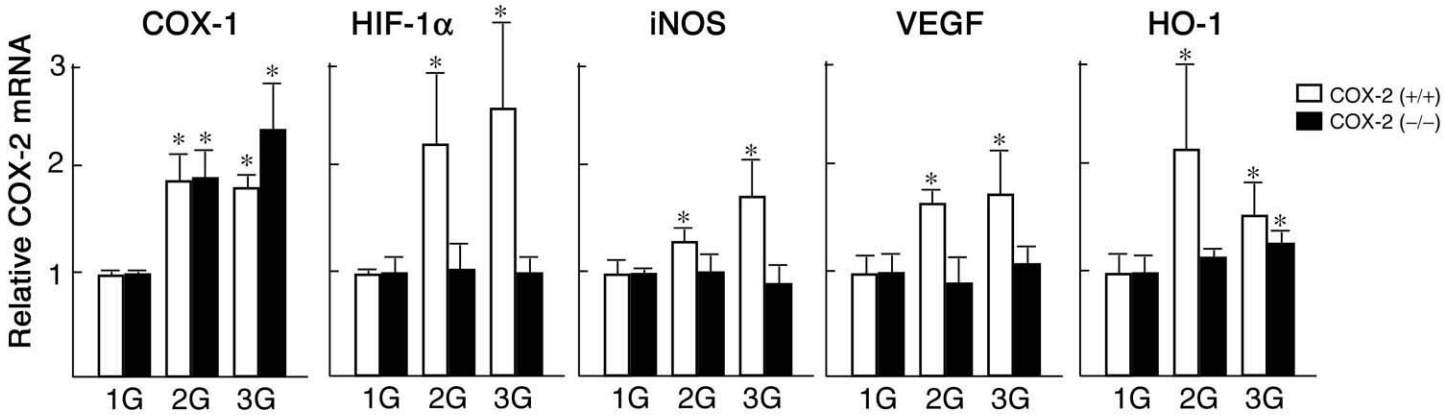


Figure 3


COX-1
HIF-1α
iNOS
VEGF
HO-1
Relative COX-2 mRNA
3
2
1
1G
2G
3G
COX-2 (+/+)
COX-2 (–/–)