Isoflurane and low-level carbon monoxide exposures increase expression of pro-survival miRNA in neonatal mouse heart

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Abstract

Anesthetics such as isoflurane are known to cause apoptosis in the developing mammalian brain. However, isoflurane may have protective effects on the heart via relieving ischemia and downregulating genes related to apoptosis. Ischemic preconditioning, e.g. through the use of low levels of carbon monoxide (CO), has promise in preventing ischemia-reperfusion injury and cell death. However, it is still unclear how it either triggers the stress response in neonatal hearts. For this reason, thirty-three microRNAs (miRNAs) known to be differentially expressed following anesthesia and/or ischemic or hypoxic heart damage were investigated in the hearts from neonatal mice exposed to isoflurane or low level of CO, using an air-exposed control group. Only miR-93-5p increased with isoflurane exposure, which may be associated with the suppression of cell death, autophagy, and inflammation. By contrast, twelve miRNAs were differentially expressed in the heart following CO treatment. Many miRNAs previously shown to be responsible for suppressing cell death, autophagy, and myocardial hypertrophy were upregulated (e.g., 125b-3p, 19-3p, and 21a-5p). Finally, some miRNAs (miR-103-3p, miR-1a-3p, miR-199a-1-5p) which have been implicated in regulating energy balance and cardiac contraction were also differentially expressed. Overall, this study demonstrated that CO-mediated miRNA regulation may promote ischemic preconditioning and cardioprotection based on the putative protective roles of the differentially expressed miRNAs explored herein and the consistency of these results with those that have shown positive effects of CO on heart viability following anesthesia and ischemia-reperfusion stress.

Keywords Anesthetic · Apoptosis · MicroRNA · *Mus musculus* · Oxidative stress · Cardioprotective

Introduction

Isoflurane is a commonly used general anesthetic that is used in pediatric patients (Wu et al. 2014; Huss et al. 2016). Compared to adults, anesthetic exposure to neonates (human and rodent) may be dangerous due to its negative impact on major systems that are developing, including the cardiovascular and nervous systems. For instance, there are major differences between adults and neonates in terms of blood-brain-barrier permeability, the ability to metabolize drugs, and proneness to hypothermia, hypotension, and hyperglycemia that could make the use of anesthetics on newborns and young

children challenging (Friesen and Henry 1986; Huss et al. 2016). Indeed, isoflurane exposure to neonatal animals with developing brains can result in neuronal apoptosis that leads to cognitive and developmental deficits (Levy et al. 2016). Similarly, retinal cells from newborn mice exposed to 1h of isoflurane (2%) have increased cytochrome C release from mitochondria, enhanced caspase activation, and an increase in the number of cells undergoing apoptosis, relative to air-treated mice (Cheng et al. 2015). However, there is reason to believe that anesthetic preconditioning (APC) can reduce myocardial injury in rat hearts exposed to hypoxia and reoxygenation (ischemia-reperfusion injury (IRI)) (Lang et al. 2013). Research to date has focused on the effects of anesthesia on mitochondrial function, since mitochondrial dysfunction can trigger apoptosis, which increases tissue damage. Isoflurane may reduce injury to organs through the activation of ERK1/2 and other kinases, including protein kinase C (PKC) (Hashiguchi et al. 2005; Lang et al. 2013). PKC-mediated phosphorylation of aldehyde dehydrogenase 2 (ALDH2), an enzyme that metabolizes toxic aldehydes,

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results in a reduction of aldehyde-protein adduct formation and reactive oxygen species (ROS) production, ultimately reducing IRI to heart tissue (Li et al. 2006; Lang et al. 2013). Furthermore, cultured cardiomyocytes from neonatal rat hearts exposed to isoflurane simultaneously with hypoxia-reperfusion stress also show decreased cleaved caspase-3 levels, decreased ROS levels, and increased cell viability compared to cardiomyocytes exposed to hypoxia-reperfusion alone (Wu et al. 2014). Therefore, isoflurane may have protective effects in the neonatal heart but few studies have been done to characterize this response.

Carbon monoxide (CO) is a versatile signaling molecule that may contribute protective effects to neonatal hearts. CO can promote vasodilation and prevent vasoconstriction, and even regulate the many processes that can prevent the rejection of a transplanted heart, including inflammation, apoptosis, and cell proliferation (Akamatsu et al. 2004). Therefore, the possibility of using low doses of carbon monoxide (CO) as a pre-treatment before cardiac insult has been explored, whether it be to prevent IRI before heart transplant or cardiac bypass surgery, or before using anesthesia, which can have detrimental effects in developing organs (Clark et al. 2003; Guo et al. 2004; Stein et al. 2005; Zuckerbraun et al. 2007; Kondo-Nakamura et al. 2010). For example, when cardiomyocyte cells are treated with ischemia reperfusion without any pre-treatments, pro-apoptotic cytochrome c is released and caspases are activated, resulting in cell death and tissue damage. Interestingly, a brief pre-treatment with CO resulted in a reduction of apoptosis including a decrease in caspase-3 cleavage (Zuckerbraun et al. 2007; Kondo-Nakamura et al. 2010).

The literature suggests that both isoflurane and CO may have protective effects on the heart before IRI through the modulation of mitochondrial metabolism and apoptosis, but to our knowledge, none has compared the cardioprotective effects of anesthesia versus low-dose CO in terms of their influence on miRNA expression. Briefly, miRNAs are small non-coding RNAs that are 21–23 nucleotides in length and can bind to mRNA transcripts with complete complementarity, resulting in their degradation, or with partial complementarity, resulting in their storage for translation at a later time (Funikov and Zatecena 2017). An individual miRNA can bind to multiple transcripts and a single transcript can be bound by various miRNAs, making this a complex and dynamic post-transcriptional regulatory system that helps control the transcriptome of a cell. Therefore, it is essential to compare how isoflurane and CO alter miRNA expression patterns in the developing heart, since individual miRNAs could influence cell death, inflammation, and other cardioprotective or injurious signaling pathways. The thirty-three miRNAs that were chosen for this study have previously been implicated in the response to anesthesia, apoptosis, and hypoxia (Supplemental Table 1). These key miRNAs were measured in the hearts of

neonatal mice to determine how isoflurane and CO differ in their regulation of cardioprotective miRNAs, with the expectation that either of these two treatments will prompt the differential regulation of miRNAs involved in cell stress. Expanding on our understanding of cardioprotective miRNAs in the developing heart could have significant clinical applications and may help physicians choose to use anesthetic or CO preconditioning before surgeries on neonatal and infant patients.

Methods

Animals

The care of the animals in this study was in accordance with NIH and Institutional Animal Care and Use Committee guidelines. Study approval was granted by Columbia University Medical Center. Six to eight-week-old breeding pairs of C57Bl/6 mice were acquired (Charles River, Wilmington, MA) and mated to yield newborn pups. On postnatal day 7, we exposed male C57Bl/6 mouse pups to air ($N = 5$), isoflurane (2 vol%) in air ($N = 5$), or 5 ppm CO in air ($N = 8$) for 1 h in a 7-L Plexiglas chamber (25 × 20 × 14 cm). The chamber had a port for fresh gas inlet and a port for gas outlet which was directed to a fume hood exhaust using standard suction tubing. Specific concentrations of CO in air (premixed gas H-cylinders, Air Products, Camden, NJ) were verified using an electrochemical sensing CO detector (Monoxor III, Bacharach, Anderson, CA). Isoflurane was delivered through a variable bypass vaporizer to the exposure chamber at a flow rate of 4 L per minute. Inhaled concentration of anesthetic was determined within the chamber (RGM 5250; Datex-Ohmeda Inc., Louisville, CO) and was maintained at 2% isoflurane for the duration of exposure. Mouse body temperature was maintained between 36 and 37°C with an infrared heating lamp (Cole-Parmer, Court Vernon Hills, IL). Animals were euthanized with a high dose of pentobarbital (150 mg/kg, i.p) immediately after exposure, and hearts were harvested straightaway and stored at −80°C.

RNA extraction

RNA isolation and miRNA amplification were performed as previously described (Biggar et al. 2018). Briefly, total RNA was isolated from whole mouse hearts ($N = 4$ –5 for each of the three conditions, weighing approximately 30 mg) using Trizol (Invitrogen, Cat# 15596018). The heart tissue was homogenized in 500 µL of Trizol reagent using a glass Dounce homogenizer. To each sample, 100 µL of chloroform was added. The samples were incubated on ice for 30 min. During this time, the samples were manually shaken every 10 min. Then, the samples were briefly centrifuged (1–2 min) at 6000 rpm

before being centrifuged at 10,000 rpm for 15 min at 4°C. The upper aqueous layer was collected and RNA was precipitated during a 10-min room temperature incubation with 250 µL of 2-propanol. Centrifugation was performed as previously described and the pellet was washed with ethanol. The samples were centrifuged again and the pellet was dried of excess ethanol for 10 min. RNA pellets were resuspended in 50 µL of autoclaved Tris-EDTA (TE) buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0). RNA purity was assessed by analyzing the 260 nm and 280 nm ratio via Take3 (Biotek, Winooski, VT, USA) analysis. RNA integrity was determined by visualizing 18S and 26S ribosomal bands on a 1% agarose gel with SybrGreen staining.

Polyadenylation and stem-loop reverse transcription

RNA samples were diluted to 700 ng/µL in a TE buffer and a polymerase. A tailing kit (Epi-Bio, Cat# PAP5104H) was used to polyadenylate the RNA such that 1 µL ATP (10 mM) and 0.5 µL *Escherichia coli* polyadenylate polymerase (2 U) were combined with 1.0 µg of total RNA in a total of 10 µL. The reactions were incubated at 37°C for 30 min, then 95°C for 5 min, before being transferred to ice. To add stem-loop adapters to RNA, 10 µL aliquots of polyadenylated RNA were incubated with 5 µL of 250 nM universal stem-loop RT primer at 95°C for 5 min and then at 60°C for 5 min. The samples were cooled on ice before reverse transcription was performed by adding 4 µL 5X first strand buffer (ThermoFisher Scientific, Cat# 18080044), 2 µL 0.1 M DTT, 1 µL 25 mM dNTP (ThermoFisher Scientific, Cat# R1121), and 1 µL M-MLV reverse transcriptase (2 U) (ThermoFisher Scientific, Cat# 18080044) to each stem-loop RNA sample. The thermocycler was programmed to 16°C for 30 min, 42°C for 30 min, followed by 85°C for 5 min. Finally, cDNA products were serially diluted and stored at -20°C until further use.

Primer design

MicroRNAs investigated in the current study were previously identified as being differentially regulated upon anesthesia treatment and ischemic or hypoxic heart damage (Supplemental Table 1). Primers for 33 key miRNAs were made as previously described in Biggar et al. (2014) using the mature miRNA stem-loop sequences obtained from miRBase database (Release 22) that were specific for miRNAs from the species *Mus musculus* (Biggar et al. 2014). Each primer pair consisted of a microRNA-specific forward primer (Supplemental Table 2) and a universal reverse primer (5'-CTCACAGTACGTTGGTATCCTTGTC-3') complementary to the stem-loop sequence. All primer sequences were synthesized by Integrated DNA Technologies (IDT Inc., Coralville, IA).

Relative microRNA quantification

Mouse-specific miRNAs were amplified and quantified using a Bio-Rad CFX96 real-time polymerase chain reaction (PCR) detection system (Bio-Rad Laboratories Inc., Hercules, CA). Reagents for PCR were prepared as described in Pellissier et al. (2006). PCR cycles were performed with the following settings: denaturation at 95°C for 3 min, followed by 45 cycles of 95°C for 10 s, and 60°C for 30 s. Twofold serial dilutions of pooled cDNA were used to test the specificity of forward miRNA primers, before quantification was done with primer sets that amplified a single melt peak indicating a single product. Several reference genes were tested (*5S rRNA*, *Snord66*, and *U6 snRNA*) but the only one that did not change expression during the test conditions (isoflurane or CO exposure) was *U6 snRNA* (5'-ACACTCCAGCTGGGGTGCTCGCTTCGG-3').

Statistical analyses

To calculate the relative expression of each miRNA, miRNAs were first quantified by the $\Delta\Delta C_t$, whereby raw C_t values were linearly transformed using the equation 2^{-C_t} to yield ΔC_t values before being standardized to the reference gene to yield the $\Delta\Delta C_t$ value (Taylor et al. 2019). Statistical analysis of gene expression and graphing was performed using Rbioplot (Zhang and Storey 2016). Relative gene expression is shown as mean $\Delta\Delta C_t \pm$ SEM, $N = 4$ -5 independent animals exposed to air, isoflurane, or CO, where control values were arbitrarily set to 1 for graphing purposes. A one-way ANOVA with a Tukey post hoc test was performed to determine statistical significance between the groups, where $p < 0.05$ was used as the threshold for significance.

Results

A number of miRNAs changed expression with either isoflurane or CO exposure with respect to air. The miRNAs that were significantly increased in hearts exposed to CO related to both the air and isoflurane treatments were miR-103-3p (2.56-fold), miR-125-5p (2.63-fold), miR-15b-5p (2.75-fold), miR-29c-5p (1.90-fold).

The miRNAs that were significantly more expressed in the CO-treated group compared to the air control but were not different compared to the isoflurane-treated group included miR-19-3p (1.41-fold), miR-199a-1-5p (2.17-fold), miR-1a-3p (1.84-fold), miR-208a-5p (1.92-fold), miR-21a-5p (2.25-fold), miR-23a-3p (2.25-fold), and miR-29a-3p (1.69-fold). The only miRNA that showed a decrease in expression during exposure to CO is miR-874-5p to 47% of the air-treated control group value. A single miRNA, miR-93-5p, increased its expression with isoflurane treatment 1.44-fold the air-treated

control group and this change was also significant compared to the CO treatment. MicroRNAs that were discussed are shown in Fig. 1; however, the statistics for all other miRNAs can be found in Supplemental Table 3.

Discussion

Volatile anesthetics can have cytoprotective effects, such as limiting damage from ischemia and reperfusion following cardiac and other surgeries, as well as myocardial infarctions and arrhythmias. Isoflurane has been shown to reduce ROS production upon reperfusion through the inhibition of complex I of the electron transport chain (Pravdic et al. 2012). However, isoflurane administration in itself tends to increase ROS formation (e.g., superoxide and nitric oxide radicals) (Kevin et al. 2005). Paradoxically, anesthetic preconditioning may rely on the prevention of ROS production as well as ROS for signaling purposes, which emphasizes our need to further elucidate the molecular pathways that govern the protective changes that are seen in the heart following anesthesia administration. Surprisingly, the results of this study showed relatively few changes in the levels of miRNAs in the hearts from isoflurane-treated mice compared to the air-treated control group. Carbon

monoxide (CO), on the other hand, seemed to invoke many changes in the expression levels of miRNAs involved in the stress response to hypoxia and ischemia-reperfusion injury (IRI).

Of all the miRNAs that were identified in the literature as being important in the response to anesthesia or low oxygen stress in the heart, only miR-93-5p increased significantly in our neonatal mice exposed to isoflurane. miR-93-5p plays a protective role in ischemia-induced cardiac injury by suppressing autophagy and cytokine expression. This is done by targeting toll-like receptor 4 (TLR4) and autophagy-related 7 (ATG7), autophagy-inducing genes. Furthermore, miR-93 may also regulate pathways related to cell death and proliferation (Abu-Halima et al. 2017; Liu et al. 2018a). miR-93 targets phosphatase and tensin homolog (PTEN), an inhibitor of PI3K/Akt pathway, such that Akt signaling is enhanced. This results in anti-apoptotic effects by phosphorylation of its downstream substrates such as BCL2-associated agonist of cell death (BAD) (Ke et al. 2016). Therefore, an increase in the expression of miR-93-5p with isoflurane exposure could inhibit the translation of genes related to autophagy, inflammation, and apoptosis that might get activated under stress.

By contrast, most miRNAs that were observed to change relative to the air-treated control group increased with CO

Fig. 1 Relative expression of key miRNAs in the hearts of isoflurane and carbon monoxide (CO)-exposed *Mus musculus* neonates, relative to control (air) levels. Relative microRNA expression was determined using RT-qPCR, $N = 4$ –5 independent RNA isolations from different animals. *U6 snRNA* was used as the reference gene. Control levels were

adjusted to 1 for graphing purposes. The one-way ANOVA with Tukey post hoc test ($p < 0.05$) was used to statistically analyze data. Different letters denote values that are significantly different from each other and shared letters indicate no statistical significance between the treatment groups (i.e., $p > 0.05$)

treatment. Both miR-29a-3p and miR-29c-5p were two such miRNAs. Many miRNAs, including the miRNA-29 family of non-coding RNAs, have roles in the regulation of cardiac hypertrophy. By targeting collagen and elastin transcripts, miR-29a-3p can inhibit fibrosis in several organs including the heart (Ślusarz and Pulakat 2015). Oppositely, the down-regulation of miR-29a-3p increases cardiac hypertrophy facilitated by nuclear factor of activated T cell 4 (NFATc4)-mediated gene transcription of skeletal actin alpha and natriuretic peptides, whereas an increase in miR-29a-3p levels results in an expected decrease in NFATc4, leading to reduced cardiac hypertrophy (Li et al. 2016a). Thus, an increase in miR-29a-3p and miR-29c-5p could be protective in the mouse heart from CO-exposed animals since these microRNAs would prevent potentially damaging changes to heart structure, including fibrosis and hypertrophy.

The levels of miR-103-3p, a miRNA implicated in several processes including cardiac cell death, regulation of the electron transport chain, and fatty acid and sugar metabolism, were also upregulated in the heart following CO treatment. Under conditions of high oxidative stress (including myocardial infarction and cell treatments with hydrogen peroxide), miR-103 inhibits cardiomyocyte apoptosis by inhibiting Fas-associated protein with death domain (FADD) and subsequent activation of pro-apoptotic caspases (Wang et al. 2015). Originally chosen for the current study because its levels were differentially regulated in rodent vasculature following treatment with different anesthetics, miR-103-3p is also upregulated in human patients with ischemic dilated cardiomyopathy (IsDC) and idiopathic dilated cardiomyopathy (IdDC) (Tutgun Onrat et al. 2018; Yao et al. 2018). Both forms of dilated cardiomyopathy and the use of anesthetics can lead to cardiac hypertrophy and muscle weakness, typically resulting in weaker cardiac contractions. Fascinatingly, CO exposure can also result in hypotension (Penney and Howley 1991), so the increase in miR-103-3p observed in CO-treated neonatal mice is postulated to be related to a change in cardiac contraction. However, one of the few studies that have analyzed the effects of miR-103 on the heart has shown that antagomiRs against miR-103/107 do not change mouse cardiomyocyte calcium flux or changes to the phosphorylation states of the proteins that regulate contractions (Rech et al. 2019). Instead, inhibition of miR-103/107 through the use of an antagomiR was associated with a decrease in mitochondria size, glycolytic capacity, and use of oxygen (Rech et al. 2019). Thus, miR-103 may be increased following CO administration as part of a mechanism that enhances energy metabolism and prevents apoptosis in neonatal cardiomyocytes.

Other miRNAs including miR-125b-3p, miR-19-3p, and miR-21a-5p were upregulated in the hearts of CO-treated mice and these miRNAs also downregulate genes involved in apoptosis, autophagy, and hypertrophy. Studies on mice have revealed that the stimulated overexpression of miR-125b is

cardioprotective (Wang et al. 2014; Bayoumi et al. 2018). Indeed, miR-125b, miR-19, and 21a have been implicated in the inhibition of cell death, leading to decreases in myocardial apoptosis and IR-mediated cell injury. Mechanisms that miR-125b may utilize include the activation of p-Akt pro-survival signaling and the inhibition of pro-cell death p53 signaling, leading to the inhibition of pro-apoptotic genes Kruppel-like factor 13 (KLF13) and BCL2 antagonist/killer 1 (BAK1). By contrast, miR-19 inhibits IR-mediated apoptosis by repressing Bcl-2-like protein 11 (BIM1), PTEN, XIAP-associated factor 1 (XAF1), phosphoinositide-3-kinase adaptor protein 1 (PIK3AP1), and BCL6 (Gao et al. 2016, 2019; Sun et al. 2017). Similar to miR-125b and miR-19, miR-21 targets genes downstream of the NF- κ B and p-Akt pathways including programmed cell death 4 (PDCD4), PTEN, and FasL to reduce cardiomyocyte death (Sayed et al. 2010; Olson et al. 2015; Qiao et al. 2015). Interestingly, miR-21 was shown to increase following isoflurane treatment in rodent heart and promote tolerance of low oxygen conditions in cardiomyocytes (Olson et al. 2015; Qiao et al. 2015). Importantly, in this study, significant changes were not observed with isoflurane treatment, which may result from fundamental differences in the responses of neonatal and adult hearts to anesthesia. Further, these results suggest CO may have an important role in the preconditioning of neonatal hearts before the administration of isoflurane, though this remains to be explored. Finally, it should be mentioned that these miRNAs have other protective functions in the regulation of autophagy, inflammation, and hypertrophy. Increased miR-125b expression can prevent NF- κ B binding activity following IR, suggesting anti-inflammatory roles for miRNA-125b. Autophagy can also be inhibited by miR-19 through the targeting of PTEN and Beclin 1 in the TGF- β II-Atg 5 pathway in cardiomyocytes (Zou et al. 2016). miR-19 may also act as an anti-hypertrophic factor, given its potent inhibition of angiotensin II (Ang-II) (a protein that induces cardiac hypertrophy) via the inhibition of phosphodiesterase 5 (PDE5A) (Liu et al. 2018b). Therefore, increases in miR-125b, miR-19, and miR-21a following CO exposure may downregulate apoptosis, autophagy, and hypertrophy, resulting in cardioprotective effects in the neonatal heart.

Originally identified as being an important miRNA in the cardiac response to volatile anesthetics (Takeuchi et al. 2014), miR-1 also has key roles in the developing heart, where it makes up a large part of the miRNA profile in the postnatally developing heart. In order to tightly control cardiac development, miR-1 inhibits a multitude of proteins involved in cardiogenesis, including the transcription factors heart and neural crest derivatives expressed 2 (Hand2) and Iroquois family of homeodomain-containing transcription factors (Irx5) (Zhao et al. 2007; Samal et al. 2019). Only 15% of mice with a deletion of one of the two miR-1 members survive and of these mice, it was demonstrated that mouse hearts with mutant miR-1 had higher Hand2 and Irx5 protein levels which

correlated with structural and functional defects in the heart, including aberrant proliferation of cardiomyocytes leading to no separation between the right and left ventricles and issues with cardiac contraction (Zhao et al. 2007). Conversely, the overexpression of miR-1 in the postnatal rodent heart leads to a decrease in cardiomyocyte proliferation (Samal et al. 2019). Thus, a 2.5-fold increase in miR-1 levels in the hearts of CO-treated animals relative to both air-treated and isoflurane-treated controls demonstrates that the protective mechanism of CO preconditioning could involve the prevention of malformations in the developing heart.

There is evidence of increased miR-199a with IRI, myocardial infarcts, and cardiac hypertrophy in both mouse and human models (Zhu and Fan 2012; el Azzouzi et al. 2013; Roncarati et al. 2014; Fan and Yang 2015; Li et al. 2017). Though the relative levels of this miRNA increase with CO treatment, similar to miR-1, these two miRNAs have opposite roles when it comes to cardiac hypertrophy. It is possible that miR-1 inhibits the effects of miR-199a on heart morphology and instead, miR-199a plays a role in energy metabolism. For instance, miR-199a can target fatty acid metabolism genes PGC1 α and PPAR δ in stressed (hypertrophic or hypoxic) heart (el Azzouzi et al. 2013; Li et al. 2016b). miR-199a-mediated changes in the expression of these two genes have been demonstrated to alter mitochondrial number and activity, as well as the switch from fatty acid oxidation to glucose utilization in the failing heart. miR-199a could therefore have an adaptive role in the CO-treated heart by promoting glycolysis for rapid energy usage in the stressed heart.

Finally, a single miRNA (miR-874-5p) decreased with CO treatment relative to both the air-treated and isoflurane-treated animals. The downregulation of cardiac miR-874-5p in CO-treated mice could be cardioprotective, based on the results of other studies. In the process of exploring the utility of sevoflurane in cardiac preconditioning before ischemia-reperfusion insult, it was determined that the downregulation of miR-874-5p could improve cardiac function by decreasing the inhibition of the Janus kinase 2/signal transducer and activator of transcription 3 (JAK2-STAT3) signaling pathway (Chen et al. 2019). This resulted in both a decrease in pro-apoptotic proteins and an increase in anti-apoptotic proteins. Though sevoflurane and isoflurane are both fluorinated anesthetics, it is interesting to observe that isoflurane exposure does not regulate this cardioprotective miRNA in neonatal mice. This emphasizes the potential benefit of administering CO before isoflurane anesthesia in neonates, since CO regulates a different set of miRNA in a cardioprotective manner.

Overall, the hearts from neonatal mice administered isoflurane or subclinical levels of carbon monoxide were found to have very different miRNA expression patterns. The assessed miRNAs were chosen based on their purported roles in the stress response during hypoxia or ischemia reperfusion, as well as in response to anesthesia administration. The

only miRNA that changed significantly in the hearts from mice given isoflurane was miR-93-5p, a miRNA identified as having roles in suppressing cell death-related processes like apoptosis, autophagy, and perhaps, inflammation. By contrast, many miRNAs were upregulated in the heart of mice exposed to low levels of CO. Most miRNAs were found to have important roles in the prevention of cell death, hypertrophy, and/or autophagy (e.g., 125b-3p, 19-3p, 21a-5p, and miR-874-5p) or for efficient energy utilization and cardiac contraction (e.g., miR-103-3p, miR-1a-3p, miR-199a-1-5p). Importantly, this experiment demonstrates that isoflurane and CO differentially regulate a different subset of miRNAs. The miRNAs that were identified as being differentially regulated could be further explored in terms of their importance in regulating cardiac function, viability, and the stress response. This study also suggests further experiments are warranted to determine how the combination of CO and isoflurane may regulate miRNA expression and downstream pathways. Results from these experiments are important to determine if the addition of CO to anesthesia could promote cardioprotection in neonatal hearts.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s12192-021-01199-0>.

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