Protective effects of pravastatin on the embryonic cardiovascular system during hypoxic development

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Abstract
The use of statins in complicated pregnancy is being considered, as they protect endothelial function in the mother and placenta. However, whether statins affect cardiovascular function in the fetus is completely unknown. Here, we have determined the effects of pravastatin and underlying mechanisms on the cardiovascular system of the hypoxic chicken embryo, a model system that permits the direct effects of pravastatin on the developing offspring to be isolated independently of additional effects on the mother and/or placenta. Chicken embryos were incubated under normoxia or hypoxia (14% O₂) from day 1 ± pravastatin (1 mg/kg/d) from day 13 of incubation (term is 21 days). On day 19 of incubation, hearts and vessels were isolated to determine changes in the cardiovascular structure and function. The data show that pravastatin protected the hypoxic chicken embryo against impaired cardiovascular dysfunction. Mechanisms involved in this protection included reduced oxidative stress, enhanced NO bioavailability, restored antioxidant defenses and normalized protein expression of RhoA in the embryonic heart, and improved NO-dependent vasodilator mechanisms in the peripheral circulation. Therefore, we show that the treatment of the chronically hypoxic chicken embryo with pravastatin from day 13 of incubation, equivalent to ca. 25 weeks of gestation in human pregnancy, has direct beneficial effects on the embryonic cardiovascular system. Therefore, pravastatin may be a candidate for human clinical translation to rescue fetal cardiovascular dysfunction in risky pregnancy.

Abbreviations: 3NT, 3 nitrotyrosine; 4HNE, 4 hydroxynonenal; Ach, acetylcholine; AWERB, Animal Welfare and Ethical Review Board of the University of Cambridge; ca., circa; CAST, Computer-Assisted Stereology Toolbox; Cu, copper; dP/dtmax, the maximum first derivative; dP/dtmin, the minimum first derivative; EC50, half-maximal response; eNOS, endothelial nitric oxide synthase; FGR, fetal growth restriction; GPx, glutathione peroxidase; GTPases, family of hydrolase enzymes that bind to the nucleotide guanosine triphosphate; HIF1α, hypoxia-inducible factor 1-alpha; HMGCoA, β-Hydroxy β-methylglutaryl-CoA or 3-hydroxy-3-methylglutaryl-CoA; HR, heart rate; K+, potassium; kPa, kilopascal; L-NAME, N omega-Nitro-L-arginine methyl ester hydrochloride; LL, left ventricular lumen; LV, left ventricle; LVPD, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; M, molar; mRNA, messenger RNA; NO, nitric oxide; NOx, nitrate and nitrite; Rac, type pyrophosphorylated intermediate essential for the actions of GTPases; Ras, type pyrophosphorylated intermediate essential for the actions of GTPases; RhoA, transforming protein RhoA or Ras homolog family member A; RL, right ventricular lumen; RV, right ventricle; SEM, standard error of the mean; SERCA, sarcoplasmic reticulum Ca²⁺-ATPase; SOD, superoxide dismutase; SNP, sodium nitroprusside; sFlt-1, soluble fms-like tyrosine kinase-1; VEGF, vascular endothelial growth factor; Zn, zinc.
1 | INTRODUCTION

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, or statins, are the most widely prescribed drugs for the prevention of cardiovascular disease, as they lower cholesterol and dramatically reduce the morbidity and mortality from coronary artery disease and stroke.\(^1\)\(^2\) It is widely accepted that statins have additional cholesterol-independent effects that are beneficial on the cardiovascular system.\(^3\) These so-called pleiotropic cardiovascular effects of statins occur via well-delineated pathways, for instance by improving endothelial function through enhanced nitric oxide (NO) production.\(^4\)

There has been accumulating clinical and scientific interest in whether statin therapy may also be protective in complicated pregnancy, conferring beneficial effects to the cardiovascular system of mother and child. There is considerable evidence that adverse conditions during pregnancy such as placental insufficiency, preeclampsia, gestational diabetes, and maternal obesity are associated with chronic fetal hypoxia, oxidative stress, and reduced NO bioavailability.\(^5\)\(^12\) The uteroplacental circulation is also highly sensitive to NO.\(^13\) Therefore, many adverse conditions during pregnancy that involve fetal hypoxia are associated with altered placental endothelial function and increased placental vascular resistance that can lead to maternal cardiovascular dysfunction as well as fetal growth restriction (FGR).\(^7\)\(^14\)

Of the wide range of statins available, pravastatin is thought to be the safest with regards to its use during pregnancy, possibly due to the hydrophilic nature of the compound.\(^15\)

Studies using animal models of adverse pregnancy have confirmed beneficial effects of pravastatin on the mother and the adult offspring.\(^16\)\(^18\) and therefore, its use in human complicated pregnancy is currently being investigated in clinical trials worldwide.\(^19\)\(^20\) However, despite the knowledge that pravastatin readily crosses the maternal-fetal interface,\(^21\) whether maternal pravastatin treatment may have direct beneficial or indeed adverse effects on the fetal circulation, independent of the mother and/or uteroplacental circulation is completely unknown.

Therefore, in this study we have isolated the direct effects of pravastatin on the growth and the cardiovascular structure and function in development complicated by chronic hypoxia, using the chicken embryo. This is the only established animal model system in which the direct effects of potential therapy on the developing fetal heart and circulation can be isolated, independent of effects on the mother and the placenta.\(^22\) Of added value, the temporal profile in the development of the gross anatomy of the heart is similar between humans and chickens.\(^22\) Because in the human clinical situation, pregnancy complicated by chronic fetal hypoxia and FGR must be diagnosed before entertaining therapy, treatment of chicken embryos with pravastatin started on day 13 of incubation (term is 21 days), equivalent to ca. 25 weeks of gestation in human pregnancy.\(^23\) This equates to a gestational age at which human FGR resulting from chronic hypoxic pregnancy can be reliably diagnosed.\(^24\) We adopted an integrative approach, combining functional experiments in the isolated heart and vasculature with molecular and cellular indices of alterations in cardiac structure and function, oxidative stress, and antioxidant defenses. The study tested the hypothesis that statin treatment has direct beneficial effects on the embryonic cardiovascular system in development complicated by chronic hypoxia via mechanisms involving reduced oxidative stress and enhanced NO signaling.

2 | MATERIALS AND METHODS

2.1 | Data, materials, and code disclosure statement

The data that support the findings of this study are available from the corresponding author. This research was approved under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical review by the University of Cambridge Animal Welfare and Ethical Review Board (AWERB).

2.2 | Animals

Fertilized Bovans Brown chicken eggs (Gallus gallus domesticus) were purchased from Medeggs (Henry Stewart & Co., Norwich, UK). This supplier delivers fertilized eggs in batches from a single day’s laying, meaning anyone egg used in the present study came from a different hen. Fertilized eggs were randomly incubated under normoxia or hypoxia (14% O2), as described previously.\(^23\) On day 13, chicken embryos from normoxic and hypoxic incubators were randomly assigned to receive pravastatin (1 mg/kg/d, Sigma-Aldrich, UK) or vehicle (100 µL water for injection) daily from day 13 to day 18 of incubation. Pravastatin was injected daily into the air cell onto the chorioallantoic membrane via a 0.5 mm hole in the eggshell. Dosing in eggs undergoing hypoxic incubation was achieved without the interruption of the hypoxia exposure in a side compartment maintained under the same oxygenation.
All treatment procedures in any egg were performed under sterile conditions.

2.3 | Dose of pravastatin treatment

The dose of pravastatin administered to pregnant women in clinical trials varies between 10 and 40 mg/d,\textsuperscript{25-27} which equates to 0.14-0.57 mg/kg/d assuming a 60kg body weight at pre-conception and a weight gain of 10kg by 25 weeks of gestation.\textsuperscript{28,29} The dose of chronic perinatal pravastatin treatment used previously in animal studies varies between 0.02 and 10 mg/kg/d.\textsuperscript{30-35} Therefore, a dose of 1 mg/kg/d was chosen for this study as a scientifically and clinically relevant dose.

2.4 | Hematocrit and growth

On day 19 of the 21-day incubation period, embryos underwent euthanasia by the dislocation of the neck and the weight was recorded for the embryo, yolk, extraembryonic membranes, chorioallantoic fluid, and the shell. Blood was collected in micro-hematocrit tubes (Vitrex, Modulohm, Denmark) for the measurement of hematocrit. The heart and brain were dissected and weighed. The heart was snap-frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\) until molecular analysis.

2.5 | Oxidative stress, SERCA, RhoA, and VEGF expression in the embryo heart

The cardiac expression of 3-nitrotyrosine (3-NT), 4-hydroxynonenal (4-HNE), and superoxide dismutase (SOD), the cardiac activity of catalase, and the total cardiac content of nitrate and nitrite (NOx) in the 19-day chicken embryo were determined using commercial kits (3-NT: ab116691, Abcam, UK., 4-HNE: E12H0203, AMS biotechnology, UK., SOD: Sigma-Aldrich, UK., Catalase: 707002 and NOx: 78001, Cayman Chemical Company, USA.). The cardiac expression of glutathione peroxidase (GPx, anti-GPx1 antibody, Abcam), SERCA (SERCA2 4388, Cell Signaling), RhoA (RhoA119 sc179, Santa Cruz), and VEGF (ab46154, Abcam) was determined by Western blot with Coomassie blue staining as a loading control\textsuperscript{36} as described previously.\textsuperscript{37} Representative gel images are included in Online Supplement Figure S2.

2.6 | Cardiac function

The heart was dissected from a separate cohort of embryos on day 19 and the basal and stimulated cardiac function was established with an isolated heart Langendorff preparation, as described previously.\textsuperscript{23} Baseline recordings were made for heart rate (HR), left ventricular end-diastolic pressure (LVEDP), and left ventricular developed pressure (LVDP). The maximum first derivative (dP/dt\textsubscript{max}) and the minimum first derivative (dP/dt\textsubscript{min}) of the LV pressure were calculated using an IDEEQ data acquisition system (version 0-2.5.0, Maastricht, Netherlands). Cardiac responses to the sympathetic β\textsubscript{1} adrenoreceptor agonist isoprenaline (Sigma-Aldrich, UK, 10\textsuperscript{−9}-10\textsuperscript{−7} M) and to the parasympathetic muscarinic agonist carbachol (Sigma-Aldrich, UK, 10\textsuperscript{−8}-10\textsuperscript{−6} M) were measured, and the ratio of the maximal HR (chronotropic) and LVDP (inotropic) responses to isoprenaline and to carbachol was calculated.

2.7 | Cardiac and aortic stereology

Another group of embryos was anesthetized with sodium pentobarbital (0.1 mL i.p. Pentoject; Animalcare Ltd, York, UK) on day 19 of incubation. An incision was made in the right atrium and the heart and the aorta were perfusion fixed at a constant physiologic pressure of 2.66kPa.\textsuperscript{38} The heart was sectioned at 1 mm thickness with a heart slicer (Zivic Instruments, Pittsburgh, USA) and quantitative analysis (7-9 sections per heart) was performed using ImageJ (version 1.46, National Institute of Health, USA) by superimposing a point grid on the cardiac sections. The volumes of the left ventricle (LV), left lumen (LL), right ventricle (RV), and right lumen (RL) were quantified using the Cavalieri principle.\textsuperscript{39} Segments of descending aorta at the level of the cardiac apex were embedded in a paraaffin block and then sectioned at 5 µm thickness. Ten consecutive sections were collected from the distal end and stained with hematoxylin and eosin. The sections were quantitatively analyzed with Computer-Assisted Stereology Toolbox (CAST version2.0, Olympus, Denmark). The person conducting these analyses was blind to the treatment group of all samples.

2.8 | Peripheral vascular reactivity

The reactivity of peripheral resistance arteries was assessed using a previously established in vitro wire myography protocol.\textsuperscript{23} Vasodilator responses to cumulative doses of sodium nitroprusside (SNP, 10\textsuperscript{−10}-10\textsuperscript{−4} M) and of acetylcholine (ACH, 10\textsuperscript{−9}-10\textsuperscript{−5} M) were determined after pre-constricting third-order femoral vessels with a sub-maximal dose of potassium (K\textsuperscript{+}, <85% of maximal response to K\textsuperscript{+} 125 mM). The partial contributions of NO-dependent and NO-independent mechanisms to the vasorelaxation within
the endothelium were determined by repeating the ACh dose-response curve with L-NAME treatment (10⁻⁵ M, 10 minutes). The difference in the area above the curve induced by ACh before after L-NAME yielded the NO-dependent contribution to the relaxation. The remaining area above the curve was taken as the NO-independent contribution to the relaxation.²³ The sensitivity (pD2) to ACh was defined as –log₁₀ (EC50). LabChart was used for data acquisition and analysis (LabChart 6.0, PowerLab 8/30; AD Instruments, Chalgrove, UK).

2.9 | Experimental design and statistical analysis

Experimental groups were assigned using a random choice generator and with the experimenters blinded to all treatments. All data are expressed as mean ± SEM. Data were checked for Gaussian distribution using the D’Agostino-Pearson normality test. Statistical comparisons were made using Two-way ANOVA, and differences isolated with the Bonferroni post hoc test. For all comparisons, statistical significance was accepted when P < .05. Statistical outliers were defined as two standard deviations ± mean, and any data points falling outside of these criteria were excluded from subsequent analyses (Graphpad prism version 5.00, San Diego, USA).

3 | RESULTS

3.1 | Effects on hematocrit and growth in normoxic and hypoxic chicken embryos ± pravastatin

These data addressed any potential protective effects of pravastatin on embryonic growth restriction induced by developmental hypoxia. Relative to normoxic embryos, hypoxic embryos showed significantly elevated hematocrit on day 19, which was not affected by pravastatin treatment (Figure 1A), meaning treated and untreated hypoxic groups experienced the same level of hypoxia. Normoxic and hypoxic embryos weighed 24.6 ± 0.8 and 17.8 ± 0.7 g, respectively (Figure 1B). The reduction in body weight in hypoxic embryos was still significant when the weight was normalized to the initial egg mass (Figure S3). Embryos exposed to chronic hypoxia had a smaller head diameter and brain weight by day 19 (Figure 1C,E) but the reduction in head size was less than the reduction in body size, such that the head diameter and the brain weight expressed relative to body weight were both increased (Figure 1D,F). In contrast, chronic hypoxia reduced the weight of the heart, lung, liver, and kidneys, which remained proportional to the effect on overall body weight (Table 1). Pravastatin treatment from day 13 of incubation had no effect on growth or organ weights in normoxic or hypoxic embryos (Figure 1 and Table 1).

![Figure 1](image_url)

**FIGURE 1** Hematocrit and fetal biometry. Values are mean ± SEM on day 19 for hematocrit (A), absolute embryo weight (B), head diameter (C), head diameter relative to body weight (D), brain weight (E), and brain weight relative to body weight (F) in chicken embryos incubated in either normoxia (N, white), hypoxia (H, black), hypoxia with pravastatin (HP, red) or normoxia with pravastatin (NP, blue). Significant (P < .05) differences are: *effect of hypoxia for post hoc test after Two-way ANOVA.
### 3.2 Effects on cardiac molecular pathways in normoxic and hypoxic chicken embryos ± pravastatin

Next, we determined any potential effects of pravastatin on molecular markers of oxidative stress (3-NT and 4-HNE) and antioxidant defenses (SOD, Catalase, and GPx), molecular markers of heart failure and altered morphogenesis (SERCA and VEGF), and on NO signaling (RhoA and NOx) in the hearts of the chicken embryos. Relative to normoxic chicken embryos, hypoxic embryos showed significant increases in the cardiac levels of 3-NT and 4-HNE, significant reductions in the cardiac activity of catalase and in the cardiac levels of SOD and NOx, without affecting the cardiac levels of GPx on day 19 of incubation (Figure 2A-C, Figure S1). The cardiac levels of NOx and SOD were significantly restored toward control levels in hypoxic embryos with pravastatin treatment (Figure 2B,C). Pravastatin did not affect any of these variables in normoxic chicken embryos. There was a significant main effect of hypoxia in enhancing levels of VEGF and decreasing levels of SERCA in the chicken embryo hearts (main effect of H, P = .0004) when compared to normoxic untreated embryos (Figure 2D,E; Figure S2). However, cardiac levels of VEGF were not different between normoxic embryos and hypoxic embryos treated with pravastatin (Figure 2D). In addition, cardiac levels of SERCA were not different between normoxic embryos and untreated hypoxic embryos (Figure 2E). Hypoxic hearts also showed enhanced levels of RhoA, which were restored toward control levels following pravastatin treatment (Figure 2F). Pravastatin in normoxic embryos also enhanced the cardiac levels of VEGF (Figure 2D).

### 3.3 Effects on cardiac function in normoxic and hypoxic chicken embryos ± pravastatin

These data determined whether any of the effects measured on molecular pathways in the hearts of chicken embryos translated to changes in basal or stimulated cardiac function. Hearts of chicken embryos exposed to developmental hypoxia showed a significantly reduced LVDP accompanied by a decrease in the dP/dt max (Figure 3A,B). In addition, the hearts of hypoxic chicken embryos had a significantly elevated LVEDP with a reduced dP/dt min (Figure 3C,D). Pravastatin treatment restored LVEDP toward control values (Figure 3C). In contrast, pravastatin did not ameliorate the reduced LVDP and dP/dt max values in the hypoxic chicken embryo (Figure 3A,B), indicating that pravastatin preferentially protected diastolic but not systolic function. In addition, both carbachol and isoprenaline had significant overall effects on heart rate and LVDP (Table S1). Values for heart rate and LVDP were decreased with increasing doses of carbachol, and values for heart rate and LVDP were increased with increasing doses of isoprenaline. Compared to normoxic embryos, the chronotropic response to the highest dose (10^-6 M) of carbachol was significantly reduced and the chronotropic response to the highest dose (10^-7 M) of isoprenaline was significantly enhanced in hypoxic hearts (Table S1). Therefore, the ratio of the maximal chronotropic responses to isoprenaline and to carbachol, an indicator of chronotropic sympathetic dominance, was increased in hypoxic embryos (Figure 3E). Similarly, the ratio of the maximal inotropic response to isoprenaline and to carbachol was enhanced in hearts of hypoxic chicken embryos (Figure 3F). Treatment

### Table 1

| Organ          | H | NP | Overall effect of | P     |
|----------------|---|----|------------------|-------|
| Brain          | 0.71 0.01 | 0.66 0.02 | Hypoxia | <.0001 |
| Heart          | 0.14 0.01 | 0.14 0.01 | Pravastatin | <.05 |
| Lung           | 0.14 0.02 | 0.12 0.02 | | |
| Liver          | 0.37 0.02 | 0.36 0.02 | | |
| Kidney         | 0.16 0.02 | 0.13 0.02 | | |
| Brain/BW       | 4.02 0.12 | 3.92 0.08 | | |
| Heart/BW       | 0.80 0.04 | 0.81 0.04 | | |
| Lung/BW        | 0.83 0.11 | 0.70 0.09 | | |
| Liver/BW       | 2.13 0.09 | 2.14 0.12 | | |
| Kidney/BW      | 0.89 0.07 | 0.78 0.10 | | |

Note: Values are mean and SEM on day 19 for absolute (g) and relative (percentage of body weight) organ weights of chicken embryos incubated in either normoxia (N), hypoxia (H), hypoxia with pravastatin (HP) or normoxia with pravastatin (NP). N = 7-10 for all data.

*Significant (P < .05) differences are:

*effect of hypoxia;

†effect of pravastatin for post hoc test after Two-way ANOVA.
with pravastatin of the hypoxic embryo restored the chronotropic and inotropic sympathetic dominance (Figure 3E,F). Pravastatin treatment in normoxic embryos had no effect on the cardiac responses to the sympathetic or parasympathetic agonists (Table S1 and Figure 3E,F).

### 3.4 Effects on the cardiovascular structure in normoxic and hypoxic chicken embryos ± pravastatin

To address any potential effects of pravastatin on the structure of the embryonic heart and major vessels, perfusion fixed tissue was analyzed. Representative mid-cardiac sections of chicken embryos are shown in Figure 4A. Exposure to hypoxia throughout development reduced the wall volume of the left ventricle (LV, Figure 4B). In contrast, the volume of the left lumen was significantly increased in the hypoxic embryo (Figure 4C). Consequently, the LV wall to lumen ratio was significantly reduced in the hearts of hypoxic embryos compared to normoxic embryos (Figure 4D). Pravastatin treatment did not prevent the remodeling of the LV in the hypoxic embryo. Areas of the lumen and wall of the aorta showed similar albeit more modest changes in embryos incubated under hypoxic conditions (Figure S3).

### 3.5 Effects on peripheral vascular reactivity in normoxic and hypoxic chicken embryos ± pravastatin

To determine the potential effects of pravastatin on endothelial function in peripheral resistance circulations, segments of the femoral arteries were studied by in vitro wire myography. The isolated femoral arterial segments from all four treatment groups relaxed in response to the endothelium-dependent agonist ACh in a dose-dependent manner. However, vessels from hypoxic embryos showed a right-shift in the curve, showing significantly impaired sensitivity (pD2) and a reduced total area above the curve (AAC), meaning impaired total relaxation to ACh and evidence of endothelial dysfunction in the peripheral vascular bed (Figure 5A,B). These effects of hypoxic incubation were prevented with pravastatin treatment (Figure 5A,B). Greater analysis of compartmental AAC revealed that the beneficial effect of pravastatin in hypoxic embryos on the ACh-induced femoral relaxation was due to a significantly enhanced NO-dependent vasodilator mechanism shown by the significantly greater area of the black histogram (Figure 5B). In addition, femoral arteries from all four groups relaxed in response to increasing doses of SNP. However, the dilator response to SNP was not affected by hypoxia or pravastatin treatment (Figure 5C).
**FIGURE 3** Cardiac function. Left ventricular developed pressure (LVDP, A), myocardial contractility (dP/dt max, B), left ventricular end-diastolic pressure (LVEDP, C), myocardial relaxability (dP/dt min, D), the ratio of the maximal heart rate responses to isoprenaline and to carbachol (chronotropic sympathetic dominance, E), and the ratio of the maximal LVDP response to isoprenaline and to carbachol (inotropic sympathetic dominance, F) of day 19 chicken embryos incubated in either normoxia (N, white), hypoxia (H, black), hypoxia with pravastatin (HP, red) or normoxia with pravastatin (NP, blue). Significant ($P < .05$) differences are: *effect of hypoxia; †effect of pravastatin for post hoc test after Two-way ANOVA. Int'; interaction of effects.
DISCUSSION

The data show that the treatment of the chronically hypoxic chicken embryo with pravastatin from day 13 of incubation, equivalent to ca. 25 weeks of gestation in human pregnancy, protects against impaired cardiac diastolic function and increased cardiac sympathetic dominance, ameliorates dilated cardiomyopathy, and prevents endothelial dysfunction in peripheral resistance circulations. Mechanisms involved in this protection include reduced oxidative stress, enhanced NO bioavailability, restored antioxidant defenses and normalized protein expression of RhoA in the embryonic heart, and improved NO-dependent vasodilator mechanisms in the peripheral circulations. Therefore, the data in this study support the hypothesis tested that statin treatment has direct beneficial effects on the embryonic cardiovascular system in development complicated by chronic hypoxia, independent of effects on the mother and the placenta. This statin-induced protection occurred despite treatment after 60% of development occurring under chronic hypoxia conditions. Therefore, pravastatin may be a candidate for human clinical translation to rescue fetal cardiovascular dysfunction, once FGR diagnosis has been possible.

The beneficial effects of statins on the cardiovascular system have been largely attributed to an improvement in NO signaling. Statin-induced inhibition of the HMG-CoA reductase blocks the mevalonate pathway, thereby preventing the synthesis not only of cholesterol, but also of pyrophosphorylated intermediates essential for the actions of the intracellular GTPases, such as Rac, Rho, and Ras. Rho and its downstream kinase negatively regulate the stability and the expression of eNOS mRNA and the activity of eNOS proteins. Therefore, inhibiting the geranylgeranylation of RhoA by statins leads to increased eNOS activity and thereby NO synthesis and availability. Statins also protect against oxidative stress directly. For instance, they stimulate the activity of Cu- and Zn-SOD via enhanced peroxisome proliferator-activated receptor activity. In addition, pravastatin protected cultured cardiomyocytes from H₂O₂-induced oxidative damage through the upregulation of catalase activity. Therefore, in the present study, prevented increases in cardiac levels of RhoA, restored cardiac NOx, improved endothelial function due to enhanced NO-dependent mechanisms, and ameliorated increases in cardiac 3-NT and 4-HNE in pravastatin-treated hypoxic chicken embryos all support mechanistic actions of pravastatin increasing NO signaling directly and/or indirectly due to its actions reducing oxidative stress. Measurement of additional protein expression levels and their phosphorylation status would have added further mechanistic insight. However, given the extensive number of analyses performed...
within this study exhausting sample availability, and the lack of availability of validated antibodies for use with chicken tissue, this was not feasible in the current study. This is a limitation.

Previous studies have also reported myocardial thinning with impaired cardiac function following developmental hypoxia in the chicken embryo, in the rodent fetus and neonate, and in young children. One candidate signaling mechanism linking developmental hypoxia and cardiac wall thinning is the interaction between HIF1α and VEGF. HIF1α is involved in cardiovascular morphogenesis, critically regulating endothelial cell migration and the development of the aortic outflow tract.50,51 HIF1α also interacts with VEGF, which is involved in the apoptotic regulation of cardiomyocytes during organ development.51 Studies in the chicken embryo have reported that the treatment of normoxic incubations with recombinant VEGF mimicked the chronic hypoxia-induced myocardial wall thinning, while the treatment of hypoxic chicken embryos with sFlt-1, a soluble receptor, and scavenger of VEGF, abolished the hypoxia-induced cardiac dilatation.48 An interesting study by Park et al52 supports a link between increased ROS with enhanced HIF1α/VEGF signaling, and inhibition of this pathway by antioxidant treatment. Therefore, improved cardiac antioxidant activity and protection against diluted cardiomyopathy by the treatment of hypoxic chicken embryos with pravastatin in the present study are all consistent with these ideas. Similarly, the induction of cardiac VEGF by pravastatin in the chicken embryo incubated under normoxic conditions associates with some degree of cardiac wall thinning in the present study. Although these effects of pravastatin appear insufficient to affect cardiac function in the normoxic chicken embryo, these data reveal an equally important translational message in the present study. Clinically, treatment with pravastatin should only be administered to pregnancies diagnosed with chronic fetal hypoxia rather than given prophylactically to all pregnancies. Further, should any treated hypoxic pregnancy be misdiagnosed, cardiac function should be monitored in the offspring.

SERCA is a pump that is localized in the membrane of the sarco- and endoplasmic reticulum. Its altered expression and activity have been implicated in heart failure in animal models and in humans for over 20 years.53-59 In line with the main effect of hypoxia in the present data, exposure to hypoxia is known to downregulate SERCA2 mRNA and protein levels in embryonic mouse cardiomyocytes via HIF1α-activation.60 Data in the present study also show that hearts from hypoxic chicken embryos had enhanced chronotropic and inotropic responses to the β1-adrenergic agonist isoproterenol, while diminished chronotropic and inotropic responses to the muscarinic agonist carbachol. Lindgren and Altimiras61 reported that chronic hypoxia also sensitizes beta-adrenergic receptors in the chicken embryo heart. This cardiac sympathetic dominance has been previously reported in adult offspring of hypoxic pregnancy in rats,62 and of obese pregnancy in mice.53 Cardiac sympathetic dominance is an adaptive response to maintain cardiac output when the function of the heart is compromised. However, sustained increases in myocardial contractility due to heightened sympathetic excitation are unsustainable and have been strongly associated with future cardiovascular dysfunction and eventual heart failure in humans.64,65 Therefore, it is of significance that treatment with pravastatin during hypoxic development in chicken embryos normalized cardiac function, preventing the need for the manifestation of this cardiac sympathetic dominant compensatory response.

In mammalian pregnancy, chronic hypoxia and oxidative stress can increase placental vascular resistance, promoting fetal growth restriction with fetal brain sparing.11,12 Accordingly, we and others have shown that supplementation with antioxidants, such as vitamin C, melatonin or MitoQ, in mammalian models of complicated pregnancy can increase umbilical blood flow and placental efficiency, thereby alleviating fetal growth restriction.13,66-71 Several studies have also reported beneficial effects of pravastatin on fetal growth secondary to reduced oxidative stress, restored vasculogenesis, and improved blood flow in the placenta in mammalian models of adverse pregnancy.31-33,72 The absence of a protective effect of pravastatin on asymmetric growth restriction in the hypoxic chicken embryo in the present study supports the concept that pravastatin improves fetal growth in compromised mammalian pregnancies at the level of the placenta. Therefore, this cannot be replicated in avian species. Alternatively, the protective effects of statins or antioxidants on fetal growth in ovine but not avian development under hypoxic conditions may reflect effects independent of those mediated at the level of the placenta. One example may be possible differences in the direct effects of pravastatin or antioxidants on fetal growth pathways between mammals and birds. However, we could not find any supporting evidence. Of interest, in avian species, eggshell porosity determines the diffusion rate of oxygen, carbon dioxide, and water vapor, consequently affecting the amount of oxygen available to the developing embryo. Seminal studies have shown that the developing avian embryo is able to increase or decrease the eggshell porosity in response to changing altitude to conserve water in ovo or to increase oxygen intake, and this occurs within the biological range of atmospheric oxygen.73 While antioxidant compounds may improve blood flow in the placenta and consequently enhance oxygen and nutrient delivery to the fetus in mammalian species,13,66-71 our hematocrit data in the present study indicate that pravastatin did not affect oxygen availability in the chicken embryo. The elevation in hematocrit level was similar in treated and untreated hypoxic chicken embryos.

A limitation of the present study is that the sex of the chicken embryo was not determined. In human clinical
practice, any current treatment of pregnancy, for instance, antenatal glucocorticoid therapy in threatened preterm birth is the same whether the offspring is male or female. Therefore, sex-dependent analysis in the present study was not an objective. However, adverse environmental conditions during pregnancy can have sexually dimorphic effects. Therefore, future studies in the chicken embryo should consider effects on the female vs the male embryo. Sexing of the chicken embryo is possible on day 19 of incubation by intra-abdominal visual analysis of gonadal symmetry. However, this analysis is highly subjective. While the basic mechanism of sex determination in birds is still unknown, there are avian homologs for most genes implicated in mammalian sex determination. Therefore, sex-dependent analysis of any measurable outcome should be possible. In addition, despite similarities in the temporal profile of the gross anatomical development of the heart between the chicken embryo and the human fetus, there are some important differences specifically in terms of timing of cardiomyocyte endowment between the species. In humans, cardiomyocyte endowment is set at around the time of birth. In chickens, cardiomyocyte proliferation can continue past hatching.  In mammals, it has been reported that the timing of exposure to hypoxia during gestation in relation to cardiomyocyte endowment has significant consequences for cardiac function. Therefore, future studies should consider investigating the effect of hypoxia and pravastatin treatment not only in the chicken embryo but also in the hatchling to address effects on cardiomyocyte endowment and maturation.

Historically, statins have been contraindicated in pregnancy mainly because there was insufficient evidence for beneficial effects to outweigh any risk. However, recent studies confirm no detrimental effects of pravastatin on the mother or fetus, including maintenance of fetal cholesterol levels, when pravastatin is administered in human pregnancy. We provide here the first evidence that pravastatin treatment in development complicated by chronic fetal hypoxia, a common adverse outcome of human pregnancy, rescues cardiac structure and function and restores endothelial function in the peripheral vasculature of the developing chicken embryo, independent of effects on the mother and/or the placenta. Future directions clearly indicate the need to design similar studies in a mammalian model, in which any additional effects of pravastatin on the mother and placenta can now be superimposed and delineated.

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CONFLICT OF INTEREST
The authors have stated explicitly that there are no conflicts of interest in connection with this article.

AUTHOR CONTRIBUTIONS
D.A. Giussani, N. Itani, and Y. Niu designed the research. N. Itani, K.L. Skeffington, C. Beck, Y. Niu, G. Katzilieris-Petras, N. Smith, and D.A. Giussani performed the research and analyzed the data. N. Itani and D.A. Giussani drafted the paper. All authors edited and approved the paper. D.A. Giussani obtained funding.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

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