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Differential expression and hypoxic regulation of adenosine receptors and TRPC channels in the developing heart.

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Abstract

Objectives: To characterize the modifications of gene expression of adenosine receptors (AR), TRPC channels, HIF-1α and iNOS during the early cardiogenesis in response to chronic hypoxia exposure. **Methods:** 4-day-old chick embryos were subjected *in ovo* to 6H, 12H and 24H of hypoxia (10% O_2). The mRNA expression was quantified by RT-qPCR. **Results:** The targeted genes were found to be expressed at mRNA level with a differential expression pattern within the heart. Hypoxia has no significant effect on mRNA expression of ARs, TRPCs channels and iNOS within the heart. By contrast, HIF-1α mRNA expression shows a tendency to be down-regulated by hypoxia. **Conclusion:** These results suggest that an intrauterine oxygen lack does not significantly affect expression of genes involved in adenosine signaling and in calcium handling by store operated channels (TRPC).

Key words: Adenosine receptors, TRPC, HIF-1α, iNOS, embryo, heart, hypoxia

Introduction

In the embryonic development, a fine oxygen sensing, supply and homeostasis are essential (1, 2). The embryo develops under physiological relative low oxygen tension (2, 3), which triggers important morphological changes and promotes growth and differentiation of blood cells, blood vessels, heart and placenta (2, 3). Due to its ability of high anaerobic glycolysis, the embryo undergoes rapid growth with high proliferation, differentiation and apoptotic rate (4). In the chick embryo, after 4 day of development although the heart is already beating, oxygen is essentially delivered by diffusion into the tissue (5). The rapid heart thickening and therefore increased diffusion distance provokes regional hypoxia (outflow tract, interventricular septum and atrioventricular junction) (6, 7). This hypoxia is an important trigger for remodeling (6) and as template for vascular growth (7, 8). The low O_2 level (less than 5%) triggers the up-regulation of hypoxia-induced responses gene. These genes are mainly activated by the transcription factor HIF-1α (Hypoxia Inducible Factor 1α) and involved in energy metabolism, cell growth, apoptosis, erythropoiesis and angiogenesis (9), essential for cell adaptation and survival (2, 10).

Pathological hypoxic insult on the developing heart

Although the embryo is able to grow under relative hypoxia, it remains extremely sensitive to oxygen deprivation. Maternal smoking, anemia, placental insufficiency, umbilical occlusion or compression, expose the embryo or fetus to in utero hypoxia (11). Adverse in utero environment in combination with genetic alteration are involved in the development of congenital abnormalities (12, 13). These diseases currently affect around 3% of the newborn (14) with 9 per 1'000 birth concerning congenital heart defect (12) and represent a major cause of infant mortality (14, 15). In order to study the impact of environment on the developing embryo, we used the embryonic chick heart as model. The *in ovo* growth allows direct effect of the environment on the embryo without maternal compensatory reaction, unlike in the mammalian *in utero* growth. In this work we focused on early embryogenesis and we use 4-day-old embryonic chick heart, corresponding to the stage 24 according to Hamburger Hamilton (HH) (16). This stage is equivalent to developmental day 11.5 (ED11.5) and day 37 (ED37) in the mouse and the human, respectively (17). The early embryogenesis (weeks 3-5 of gestation in human) represents a period of high cardiac susceptibility to adverse environment (18).

The chronic lack of oxygen induces development alteration and injury to vital organs. The morphological and functional impact depends on the intensity, the duration and the timing of the hypoxic exposure (13). Chronic hypoxia globally affects the embryo independently from the model with reduced weight and survival (19, 20). In the chick embryo, chronic hypoxic exposure from the beginning of the incubation is lethal (8). Grabowski and al. have observed and described the morphological functional modification of the heart and cardiovascular system in the 3-day old chick embryo(21). They found dilated heart, slow heart rate, arrhythmias and cardiac arrest, enlarged blood vessels, high blood pressure, decreased hematocrit and hemorrhages (21). Stage 24HH chick embryo exposed to chronic hypoxia presents morphological malformation like cardia bifida and looping defect (22). The cardiovascular system is also functionally affected with impaired systolic function and inappropriate hemodynamic response to stress (19). The electrophysiological modifications observed on the 4-day-old beating heart show atrio-ventricular conduction disturbances and arrhythmias such as atrial ectopy (23). The hypoxia-induced alterations are based on activation and repression of molecular pathway and response genes, some of these important genes are described below.

The molecular pathway involved in the response and adaptation to hypoxia.

1. *HIF-1α, hypoxia inducible factor 1α*

HIF-1 is a ubiquitously expressed oxygen-sensing transcription factor responsible for oxygen (O_2) homeostasis (2, 24). The dimeric HIF-1 protein is composed of a constitutively expressed β-subunit and an O_2-regulated α-subunit. In presence of O_2, the hydroxylation and the binding to von Hipple-Lindau (VHL) protein leads to proteasomal degradation of HIF-1α (24). On contrary, under hypoxic condition the degradation is inhibited, HIF-1α is stabilized and translocated into the nucleus for hetero-dimerization with the β-subunit and transcriptional activity (9, 10). The HIF complex binds to specific hypoxia responsive element (HRE) on dozen of targets genes involved in the response and adaptation to hypoxia (9). During embryogenesis HIF-1α is required for the proper formation of the embryo and the heart (25). In the early mouse embryo from ED9.5, HIF-1α is highly expressed at mRNA level in the heart, especially in the ventricular wall (26). The myocardial hypoxia witch activates HIF-1α, triggers angiogenesis and remodeling of the outflow tract (25, 27). HIF-1α plays a central role in the embryonic development under physiological low O_2 tension. The regulation of HIF-1α by pathological hypoxic environment is still not clearly understood. Many evidences have pointed the predominant post-translational regulation acting on protein stability and ARNT dimerization (28-30). The avian embryo exposed to hypoxia shows a HIF-1α protein up-regulation (31). The impact at mRNA level remains more unclear and differs between studied models and tissue. In the adult mice hypoxia has no effect on mRNA expression (29). In rat brain, hypoxic significantly up-regulates HIF-1α mRNA expression (32). Supplemental works are needed to specify HIF-1α expression and regulation in the embryonic heart.

2. *Adenosinergic system*

In the early embryo, there is no autonomous cardiac innervation. The nucleoside adenosine plays a central role in the cardiac function as major humoral regulator (33, 34). Adenosine is produced by catabolism of ATP or ADP by ectonucleotidases (35). The cellular level of adenosine under physiological conditions is in the nanomolar (nM) range (10 to 100 nM) and rises about 100-fold in stress conditions like ischemia, hypoxia or increased tissue activity (36). Adenosine acts on four receptors respectively named A_1 adenosine receptor (A_1AR), A_{2a} adenosine receptor (A_{2a}AR), A_{2b} adenosine receptor (A_{2b}AR) and A_3 adenosine receptor (A_3AR). These receptors are G-protein coupled receptors with specific ligand affinity, A_1AR and A_{2b}AR showing the highest and lowest affinity, respectively (36). A_1AR and A_3AR interact

with G_i and G_o protein, whereas $A_{2a}AR$ and $A_{2b}AR$ interact with G_s and G_q protein (35). The A_1AR is the first receptor expressed during embryogenesis. In the gastrulating chick embryo (stage 4 HH) A_1AR is present in the heart forming region (22) so as in the rat myocardium at gestational day 8 (33). At this early stage in the mammalian, A_1AR is mainly involved in the pacemaking function and its activation provokes slowed heart rate to asystolia (37). During intra-uterine hypoxic stress, A_1AR provides protection to the mammalian embryo and is needed for adequate response (38, 39). On contrary in the chick embryo at stage 4 HH exposed to hypoxia, A_1AR mediates the deleterious effect of hypoxia and induces cardiac malformations (22). Furthermore in the 4-day-old embryonic chick heart, the A_1AR activation is proarrhythmogenic through ERK, PLC-PKC activation and therefore $[Ca^{++}]_i$ level modification (40). The $A_{2a}AR$, $A_{2b}AR$ and A_3AR are also expressed in the embryonic chick heart at ED4 (40) but their specific function in the heart remain unclear. The $A_{2a}AR$ and $A_{2b}AR$ expressed in the ventricle mediated enhanced contractility in response to different adenosine level (41, 42). The A_3AR overexpression in the ED8.5 mice embryo induces lethality (43) and in the adult mice heart leads to conduction disorders and cardiomyopathy (44).

During hypoxic stress the adenosinergic system is widely activated with important adenosine production and activity on the receptors. However little is known about potential hypoxic regulation of the AR expression. Some studies have shown regulation of the AR by hypoxic exposure but with considerable variability depending on studied tissues and conditions. After chronic hypoxic exposition, immortalized cell model of smooth muscle shows an up-regulation of A_1AR (45), on contrary C6 glioma cells show A_1AR down-regulation (46). The A_2AR ($A_{2a}AR$ and $A_{2b}AR$) are up-regulated by hypoxia in the human endothelial and smooth muscle cells (47), in fetal chromaffin-derived cell line (48) and in placental villous explant (49). Modulation of the receptors in the embryonic heart is still unknown.

3. Transient Receptor Potential Canonical (TRPC) channels

It is well established that cardiac function is tightly controlled by voltage-dependent Ca^{2+} channels in embryonic and adult heart but recent evidences suggest that the TRPC channels family may regulate cardiac activity. The TRPC channels are part of the Transient Receptor Potential superfamily. In mammalian, the TRPC family includes 7 isoforms (TRPC1 to -7) and they assemble as homo or hetero-tetramers around a central ion selectivity pore to form a great variety of cation-selective channels (50). They mainly regulate intracellular calcium level (51). They are divided into 2 groups based on structural and functional similarities:

TRPC1/4/5 and TRPC3/6/7. TRPC1/4/5 channels are proposed candidate subunits of Store-Operated channels (SOCs). These types of channels are activated by the Ca2+ stores depletion and IP3-dependent mechanisms (52). TRPC3/6/7 are activated by G-protein coupled receptor via PLC/diacylglycerol (DAG) pathway (51, 53).

In heart, they are involved in numerous physiological and pathological processes like arrhythmias, hypertrophy, heart failure and apoptosis (51, 54-56).

In the embryonic chick heart, they are all expressed except TRPC2 at the mRNA and protein level (57). The crucial role of TRPC channels, especially TRPC3 isoform, in regulating the cardiac electrical activity in the embryonic heart, has been demonstrated in our laboratory. Indeed, the inhibition of these channels induced atrioventricular block especially Wenckebach phenomenon (Mobitz type I), widening of the QRS complex and prolonged QT duration (57). More recently, the laboratory showed that TRPC3 is involved in the arrhythmias induced by A_1AR activation (58). Therefore TRPC3 could be implicated in the adenosine regulated hypoxic-response. All the previous data suggest that TRPC could be regulated by hypoxia in our model. Interestingly, it has been demonstrated that hypoxia increases TRPC1 and TRPC6 mRNA expression by activation of the transcription factor HIF-1α in PASMC cells (pulmonary arterial smooth muscle cell) (53). This up-regulation is responsible for the development of hypertrophic cell and hypertension (59-62).

4. iNOS, inducible nitric oxide synthase

Nitric oxide (NO) is an intracellular signaling molecule, produced by NO synthase (NOS) from L-arginine. There are three NOS isoforms, the nNOS (neuronal) and eNOS (endothelial) constitutively expressed and the iNOS, the inducible forms expressed in the heart under stress, hypoxic or inflammatory conditions (63). In the early murine embryonic heart iNOS is highly expressed under physiological condition, from stage ED11 to stage ED14.5, and decrease to undetectable expression at birth time (64). Similar expression level is observed in the embryonic chick heart, with peak expression at stage 34 HH (65). Activation of iNOS produces a large amount of NO required for embryonic development (66). Furthermore iNOS plays an important role in the heart contractile function and angiogenesis (65, 67). In fetal heart from ginea pig, iNOS mRNA and protein are up-regulated by chronic hypoxia in association with HIF-1α increase. iNOS may play a specific role in the developing embryo during hypoxic stress (63). The period of high iNOS mRNA expression coincides with a phase of important physiological hypoxia in the embryo (7). This fact suggests the possibility of a hypoxic regulation or stimulation of iNOS expression.

This work aimed to characterize the expression pattern of the adenosine receptors, TRPC channels, iNOS and HIF-1α in the different parts of the chick embryonic heart. In order to better understand the pathophysiological processes involved in the embryonic response to hypoxia we aimed to evaluate the potential hypoxic regulation of the mRNA expression of the TRPC channels and AR. Some genetic changes in response to adverse environment persist until adulthood and are involved in the development of cardiovascular diseases. The embryonic and fetal adaptation to such conditions might induce permanent morphological, functional and genetic modifications (3, 68, 69).

Materials and methods

Embryo preparation. Fertilized chicken eggs were obtained from Lohman Brown hens. The eggs were incubated during 96 hours, corresponding to the Hamburger-Hamilton stage 24 (24 HH) (16), in an incubator maintaining an atmosphere at 38°C and 95% of humidity. To analyze the time-dependent impact of hypoxia, the eggs were divided into two groups: the normoxic control group was incubated at 21% oxygen, and the hypoxic group was placed in a hypoxic chamber at 10% oxygen for the last 6 hours, 12 hours and 24 hours of incubation (6H, 12H and 24H). At 4-day of development, the hearts were carefully excised and dissected into atria, ventricle and outflow tract (as shown in *Figure 1*) then these parts were separately immersed in 200µL RNAlater reagent (Quiagen) and stored at -80°C until RNA isolation. The embryo and the heart were manipulated under binocular glass with micro-instrument in a standard buffered medium ((in mmol/l) 99.25 NaCl; 0.3 NaH_2PO_4; 10 $NaHCO_3$; 4 KCl; 0.79 $MgCl_2$; 0.75 $CaCl_2$; 8 D+Glucose) cooled at < 4°C to prevent RNA degradation. In order to obtain enough isolated RNA from the reverse transcription, at least ten of each dissected part were pooled together. All the following procedures were assessed in parallel, under the same condition, for the normoxic and hypoxic groups.

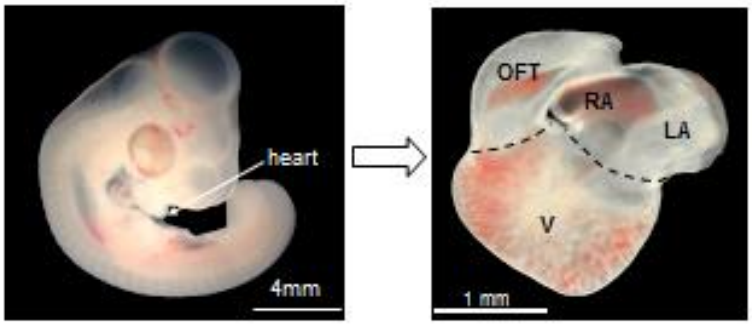

Figure 1. 4-day-old chick embryo and dissected heart; ventricle (V), left and right atria (LA and RA, respectively) and outflow tract (OFT). Dotted line represents the dissection line.

RNA extraction. RNA extraction from the atria, ventricle and outflow tract tissue from 4-day-old chick embryo was performed using TRIzol reagent (Invitrogen, Basel, Switzerland). Briefly, the RNAlater was carefully removed, the heart tissue samples were homogenized in 500µL TRIzol reagent and incubated at room air temperature (5min), and then 100µL of Chloroform (Sigma-Aldrich, Buchs, Switzerland) were added for phase separation. The samples were vigorously shaken and incubated at room temperature (5min) before centrifugation (15min, 12'000g, 4°C). To precipitate RNA, 250µL of Propanol (Sigma-Aldrich) were added to the upper aqueous phase containing the RNA and incubated at room air temperature (10min) and then centrifuged (10min, 12'000g, 4°C). The pellet was washed with 500µL of 75% ethanol, centrifuged (5min, 7'500g, 4°C) and dried during 5 minutes. The RNA pellet was finally resuspended in 20µL of RNase-free water (Qiagen, Hombrechtikon, Switzerland) and incubated during 10-15 minutes in heat block (55-60°C). The RNA was then directly used for downstream reverse transcription or store at -80°C. The quantification of extracted RNA was determined by Nanodrop spectrophotometry (Thermo Scientific). The purity was assessed with the two absorbance ratio A260/280 and A260/280. A230, A260 and A280 represented the absorbance of organic compounds, protein and RNA/DNA respectively.

Reverse transcription. GoScript™ Reverse Transcriptase (Promega Corporation, Madison WI, USA) and the related Promega protocol were used. Briefly, 1µg of mRNA, 1µL Random primers (500µg/ml) and RNase-free water were mixed in a total volume of 8µL, heated 5 minutes at 70°C and then directly chilled out on ice at least 5 minutes. Then 4µL of 5X Reaction Buffer, 2µL of $MgCl_2$ (25mM), 1µL of dNTP (10mM), 1µL of Recombinant RNasin Ribonuclease Inhibitor (40u/µL), 3µL of RNase-free water and 1µL GoScript™ Reverse Transcriptase (200U/µL) were added to each sample for a final mixture volume of 20µL.

Thermal cycling was: 5min at 25°C, 1H at 42°C, 15min at 70°C, then the samples were cooled and store at -80°C or directly used for the quantitative PCR.

Real time quantitative PCR (RT-qPCR). Quantitative polymerase chain reaction was carried out using SYBR-Green and ViiA 7^{TM} device (Applied Biosystems, Warrington, UK). The expression of A_1AR, A_{2a}AR, A_{2b}AR, A_3AR, TRPC1, TRPC3-7, HIF-1α and iNOS were analyzed. The list of primers used (Microsynth AG, Balgach, CH) is shown in the *Table 1.*

Gene	Forward	Reversed	Annealing Temperature
A_1AR	5'GTG GGA CTC CCC AAT GTT C3'	5'ACT CCG AGT GGC TGA CAT TCA G3'	60°C
A_{2a}AR	5'GCA GCT CAA GCA GAT GGA GAA C3'	5'TGG CTG CGT GGA CTT CCT T3'	60°C
A_{2b}AR	5'GCC ATG AGT GGT TGT CCT AAC T3'	5'TCT CAA AGA GAC ACG AAA AGC A3'	57°C
A_3AR	5'CCA CAT GAC ATT AAG CCC AAA A3'	5'AGC GAC AAC CAA GCC AAA TT3'	58°C
TRPC1	5'CGA CAT TCC AGG TTT CGT CTT3'	5' ATT GGA TCC TCC TCC GTC AA3'	58°C
TRPC3	5'GGT ACT TGG TAT GAT GTG GTC AGA A3'	5' CAA CAT CCC AAA ATC CAA AAC A3'	58°C
TRPC4	5'CTC AAC ATG CTA ATA GCC ATG ATG 3'	5'TCC GAG CAA ACT TCC ATT CA3'	58°C
TRPC5	5'TGC CCT GGG TTC TAG GTT TAT3'	5' CCA TTA GAT TCC ACC AGT CAT GTA C3'	58°C
TRPC6	5' CTC CTA CTA TCT GGG AGC AAA ACA3'	5'ATA TAG CCC AGA ACA ACG TCT TGA3'	58°C
TRPC7	5'GGA GGAGTC CAA AAC CTT GAA C3'	5'TGC TCA TTC CCT ACA GCT AAC TGT3'	58°C
iNOS	5'TCT TCC AGC TAA AGA GCC AAA AG3'	5'CAC GTC CAA TGT CTG TTG TTC A3'	60°C
HIF-1α	5'CAC ACC ATG ATA TGT TCA CGA AA3'	5'AAC CCA GAC GTA GCC ACC TT3'	57°C
GAPDH	5'GAC ACT TCA AGG GCA CTG TCA3'	5'GGT CAC GCT CCT GGA AGA TAG3'	57°C
GAPDH	5'AAG CAG GAC CCT TTG TTG GA3'	5'GGA ACA GAA CTG GCC TCT CAC T3'	58°C
GAPDH	5'AAG GGT GGT GCT AAG CGT GTT 3'	5'TTC TCA TGG TTG ACA CCC ATC A3'	60°C

Table 1. Nucleotide sequence of PCR primers for amplification of adenosine receptors (AR), transient receptor potential canonical (TRPC) channels, iNOS (inducible nitric oxide synthase), HIF-1α (hypoxia inducible factor 1α) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase), and the corresponding annealing temperature.

The amplification was performed in a total reaction volume of 10µL with PCR mix contained SYBR-Green (Applied Biosystems), forward and reversed primers (Microsynth), cDNA and RNase-free water (Quiagen) at concentration and quantity detailed in *Table 2*.

Gene	SYBR-Green 2X (µL)	[Primer] (nM)	cDNA quantity (ng)
A_1AR	5 µL	300 nM	5 ng
A_{2a}AR	5 µL	200 nM	5 ng
A_{2b}AR	5 µL	400 nM	50 ng
A_3AR	5 µL	300 nM	25 ng
TRPC1	5 µL	300 nM	25 ng
TRPC3	5 µL	200 nM	5 ng
TRPC4	5 µL	300 nM	25 ng
TRPC5	5 µL	200 nM	25 ng
TRPC6	5 µL	300 nM	25 ng
TRPC7	5 µL	300 nM	25 ng
iNOS	5 µL	300 nM	10 ng
HIF-1α	5 µL	200 nM	5 ng
GAPDH	5 µL	150 nM	20 ng

Table 2. Quantities and concentrations of the PCR mix products. Final volume of 10µLper well. Primers were prepared from a 10µM initial solution. cDNA was taken from the 50ng/µL final RT concentration.

RT(-) samples were used as a negative control and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal control for all the experiences. Cycling conditions were as follows: 2min at 50°C, 10min at 95°C followed by 40 cycles of 95°C for 15sec, then 60°C for 1min. For all reactions, each sample was triplicated on 96-well plates with optical sealing tape, and signals were measured with sequence detector system software (Applied Biosystems).

Data analysis. The data were analyzed with the delta delta Ct method as detailed (70):

$$- \Delta\Delta Ct = - (Ct_{target} - Ct_{ref})_{hypoxia} - (Ct_{target} - Ct_{ref})_{normoxia}$$

$$\text{then } 2^{-\Delta\Delta Ct}$$

The reference gene ("ref") is the GAPDH gene. The $2^{-\Delta\Delta Ct}$ expressed the mRNA fold changes expression of the hypoxic condition relative to the normoxic conditions.

Statistical analysis. The data are presented as mean ± the standard error of the mean (SEM). Statistical analyses and graphs were performed with GraphPad Prism (GraphPad Software Inc., San Diego, CA). The non-parametric Man-Withney test was applied to each assay for statistical comparison. Statistical significance was considered by $P< 0.05$ (*<0.05, **<0.01, ***<0.001).

Results

This work focused on the mRNA expression in the 4-day-old embryonic chick heart. The expression pattern of the adenosine receptors, TRPC channels, iNOS and HIf-1α in the different chambers of the heart were analyzed. The effects of chronic hypoxia on the mRNA expression were quantified after various hypoxic time exposures.

GAPDH expression pattern. The GAPDH was equally expressed in the atria, the ventricle and the outflow tract, and was stable under chronic hypoxia exposure except after 24 hours of hypoxia exposure (see annexes). Because of this stability, GAPDH was used as internal control gene for all the experiences. Therefore after 24 hours of hypoxia, the GAPDH mRNA expression increases in the ventricle (data not show) and an important variability in the results was observed. That's why the results at 24 hours of hypoxia in the ventricle are not shown.

Distribution of adenosine receptors mRNA expression in the heart. The four adenosine receptors were detected by RT-qPCR at mRNA level in the embryonic heart. These receptors are differentially expressed. The direct comparison of the expression level is not possible because of different primers specificity and different concentrations of cDNA used. The Ct values show that A_1AR (mean Ct = 31), A_{2a}AR (mean Ct = 27) and A_3AR (mean Ct = 28) are well expressed. The A_{2b}AR (mean Ct = 39-40) is barely detectable and shows important variability. According to this, the data of A_{2b}AR are not discussed here (see

annexes).The distribution in the heart of each receptor is different (*Figure 2*). The A_1AR is twice more expressed in the atria and ventricle than in outflow tract, but with higher expression in the atria than in the ventricle. The A_{2a}AR is predominantly expressed in the outflow tract with a two to three-fold higher expression than in the atria and the ventricle, respectively. The A_3AR is equally present in the atria, the ventricle and the outflow tract.

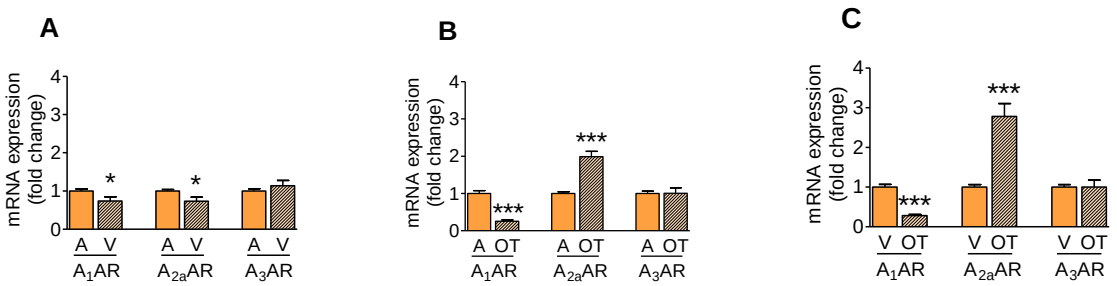

Figure 2. mRNA expression pattern of the adenosine receptors in 4-day-old embryonic chick heart. Quantitative RT-PCR was performed on isolated atria (A), ventricle (V) and outflow tract (OT). **(A)** mRNA expression in ventricle vs. in atria. **(B)** mRNA expression in outflow tract vs. in atria and **(C)** mRNa expression in outflow tract vs. ventricle. A_1AR n=9-10; A_{2a}AR n=9-11; A_3AR n=10-11 determinations. *P< 0.05

Effect of chronic hypoxia on the adenosine receptors mRNA expression. The embryos were exposed to 6H, 12H or 24H of chronic hypoxia. None of these exposures significantly modified adenosine receptors mRNA expression. The small number of analyzed assays did not allow to observe significant modification and only tendencies can be interpreted from these results. The expression of A_1AR shows a time dependent progressive downward trend in the atria, when in the outflow tract the decreased expression trend at 6H get reversed after 24H of hypoxia (*Figure 3A-C*).The A_{2a}AR expression level remains clearly stable in the atria and the outflow tract, while in the ventricle the receptor tends to be up-regulated (*Figure 3D-F*). The A_3AR expression is not modified by hypoxia with the exception of ventricular mRNA expression after 12H of hypoxia where tendency to down-regulation is observed (*Figure 4*).

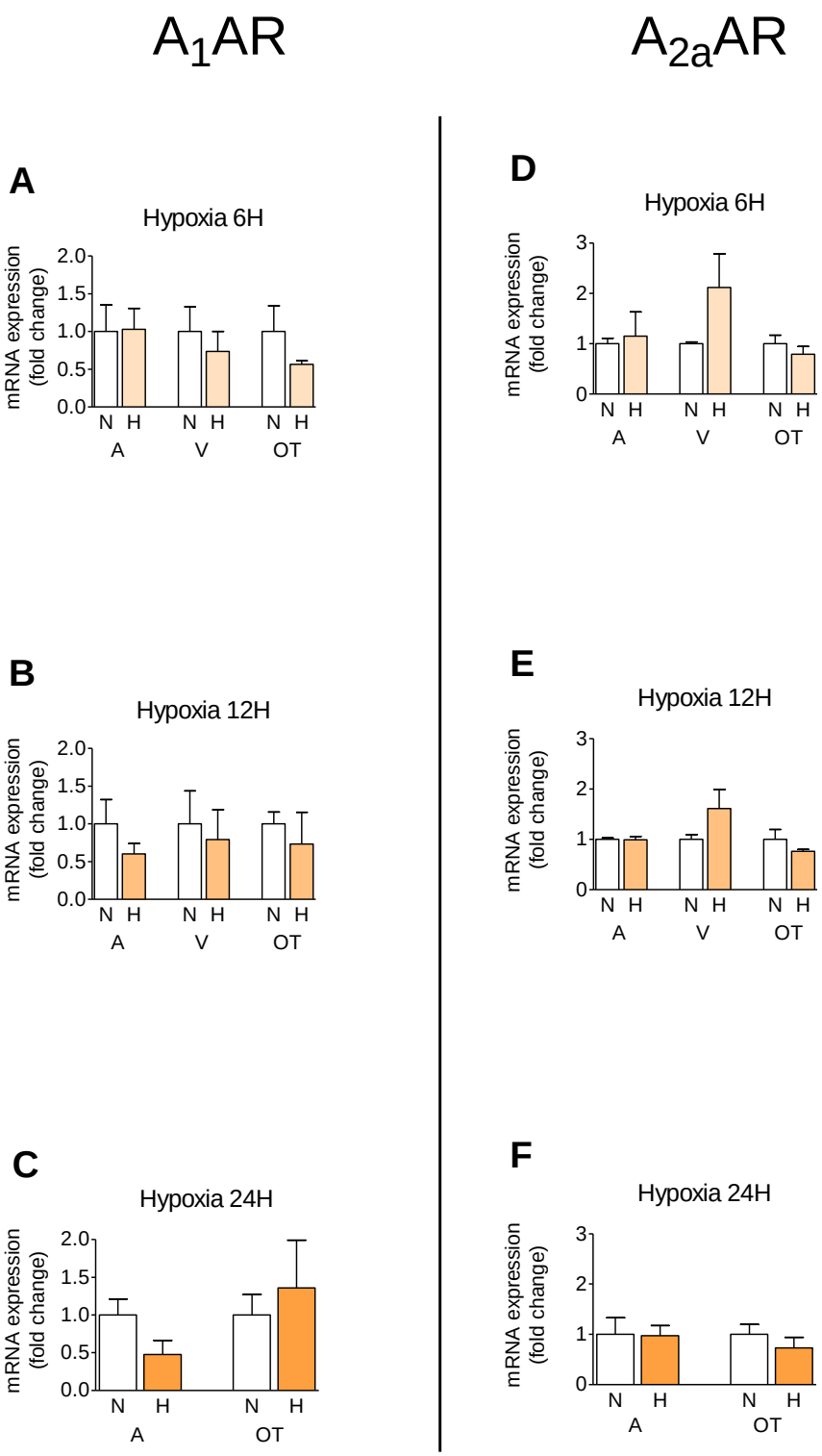

Figure 3. Time-dependent effect of hypoxia (6H, 12H and 24H) on A_1AR **(A, B, C)** and A_{2a}AR **(D, E, F)** mRNA expression in the 4-day-old embryonic chick heart (ED4). The mRNA gene expression was quantified by RT-qPCR performed on isolated atria (A), ventricle (V) and outflow tract (OT). A_1AR n=3-4; A_{2a}AR n=3-4 determinations.

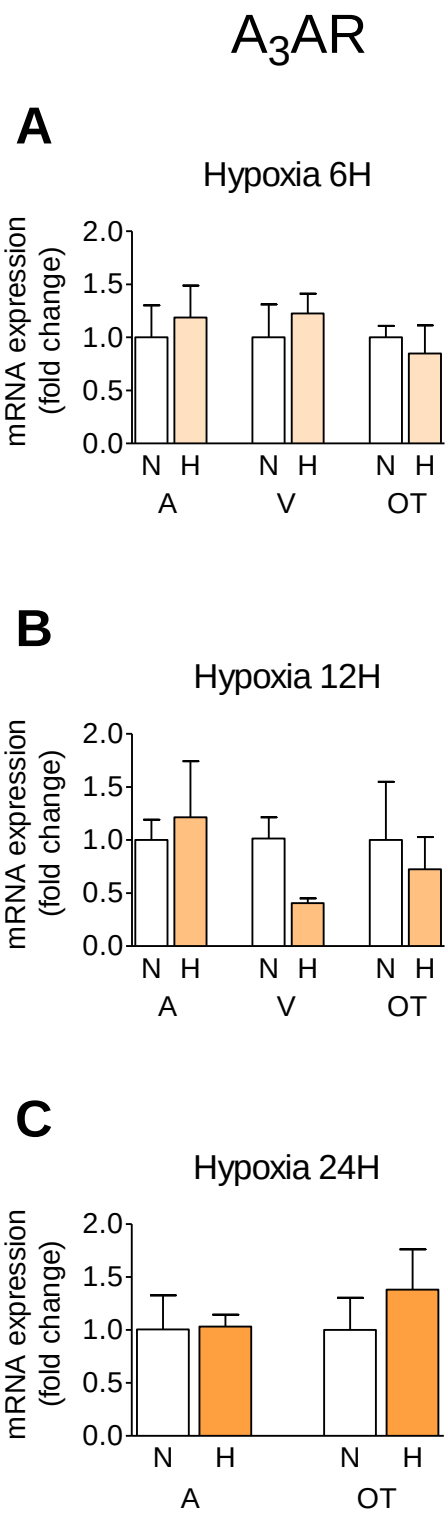

Figure 4. Time-dependent effect of hypoxia (6H, 12H and 24H) on A_3AR **(A, B, C)** mRNA expression in the 4-day-old embryonic chick heart (ED4). The mRNA gene expression was quantified by RT-qPCR performed on isolated atria (A), ventricle (V) and outflow tract (OT). A3AR n=3-4 determinations.

Distribution of the TRPC channels mRNA expression in the heart. The TRPC1 and TRPC3 to TRPC7 channels are expressed in the 4-day-old developing chick heart and showed specific expression pattern (*Figure 5*). The TRPC1 and TRPC7 channels are equally expressed in atria, ventricle and outflow tract. The TRPC3, TRPC4 and TRPC5 channels have similar distribution with predominant expression in the outflow tract, up to four times more than in atria and ventricle for TRPC4 channel (*Figure 5B, C*). The TRPC6 channel is predominantly expressed in the atria. The ventricular expression is twice less important than the atrial and conotruncal one. According to the Ct, TRPC1 (mean Ct = 25) seems to be more expressed than the other TRPC channels (TRPC3 mean Ct = 29, TRPC4 mean Ct = 30, TRPC5 mean Ct = 30, TRPC6 mean Ct = 29, TRPC7 mean Ct = 31). In conclusion, most of the channels are predominantly expressed in the outflow tract and the less in the ventricle.

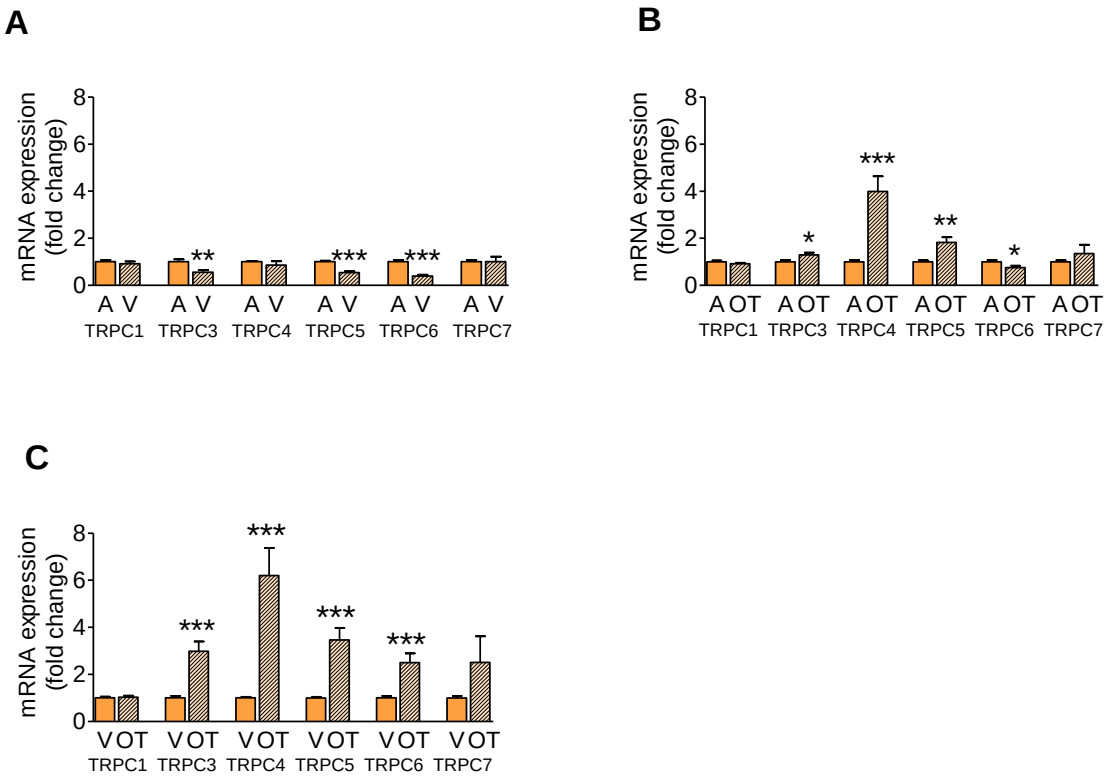

Figure 5. TRPC mRNA expression pattern in 4-day-old embryonic chick heart. Quantitative RT-PCR was performed on isolated atria (A), ventricle (V) and outflow tract (OT). **(A)** mRNA expression in ventricle vs. in atria. **(B)** mRNA expression in outflow tract vs. in atria and **(C)** mRNa expression in outflow tract vs. ventricle.TRPC1 n=9-13; TRPC3 n=8-10; TRPC4 n=10-12; TRPC5 n=9-11; TRPC6 n=10; TRPC7 n=9-10 determinations. **P*< 0.05

Effect of chronic hypoxia on the TRPC channels mRNA expression. Like observed in the basal expression, some channels show similar expression pattern and modifications. The TRPC1 (*Figure 6A, B*) and TRPC6 (*Figure 8A, B*) channels express an overall trend to decrease after 6H and 12H of hypoxia. After 6H and 12H of hypoxia, the TRPC4 (*Figure 7A, B*), TRPC5 (*Figure 7D, E*) and TRPC6 (*Figure 8A, B*) show a similar tendency of mRNA down-regulation in the ventricle. The TRPC3 (*Figure 6D-F*) and TRPC7 (*Figure 8D-F*) mRNA expression shows no consistent tendency over the time after hypoxic exposure. Despite the clear tendencies no significant changes were observed. Further assays should be carried out in order to confirm or deny the tendencies.

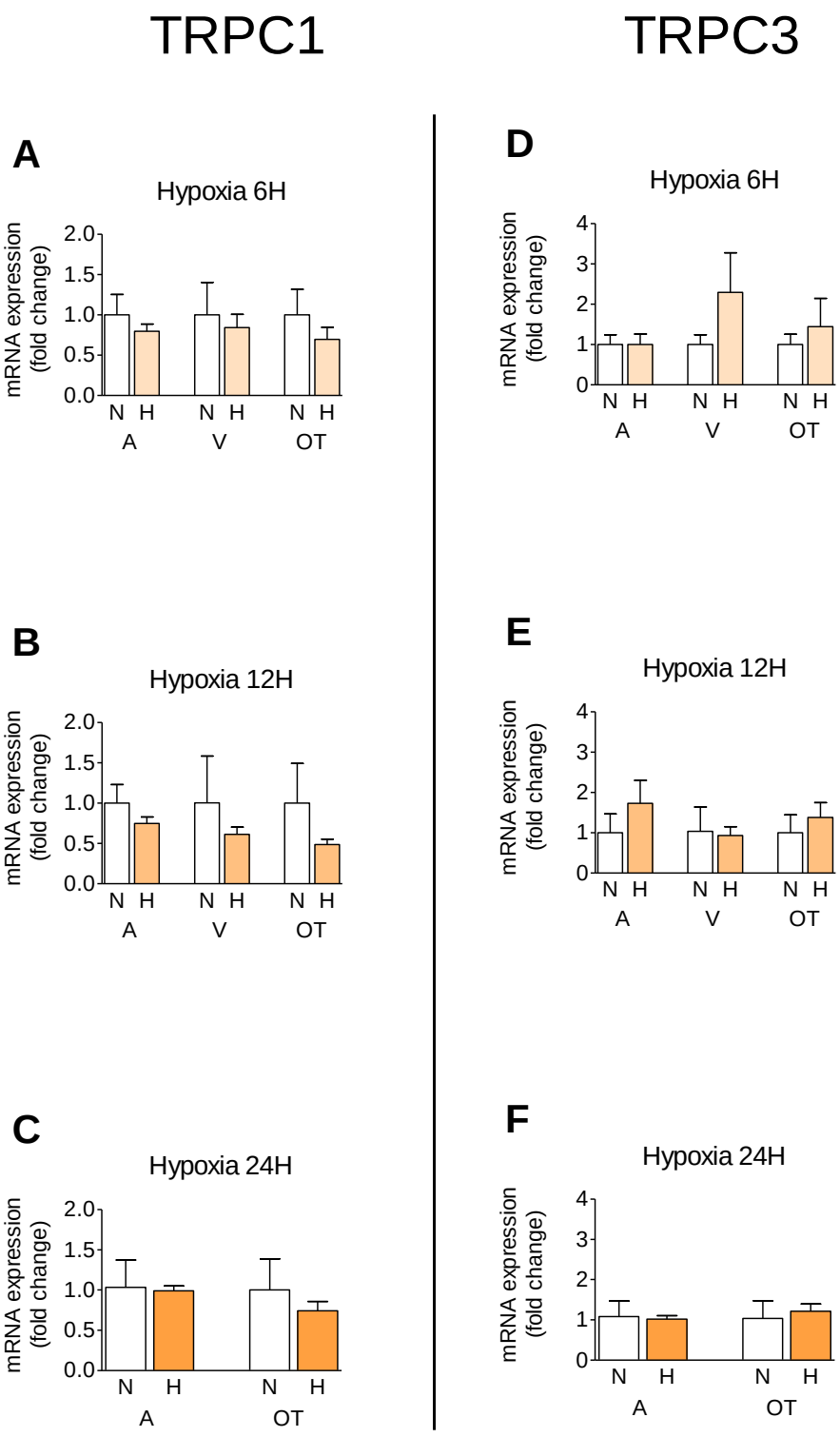

Figure 6. Time-dependent effect of hypoxia (6H, 12H and 24H) on TRPC1 **(A, B, C)** and TRPC3 **(D, E, F)** mRNA expression. The mRNA expression was quantified by RT-qPCR performed on isolated atria (A), ventricle (V) and outflow tract (OT) from 4-day-old chick embryo (ED4) exposed to normoxic (N) (control group) and hypoxic conditions (H). TRPC1 n=3-4; TRPC3 n=3-4 determinations.

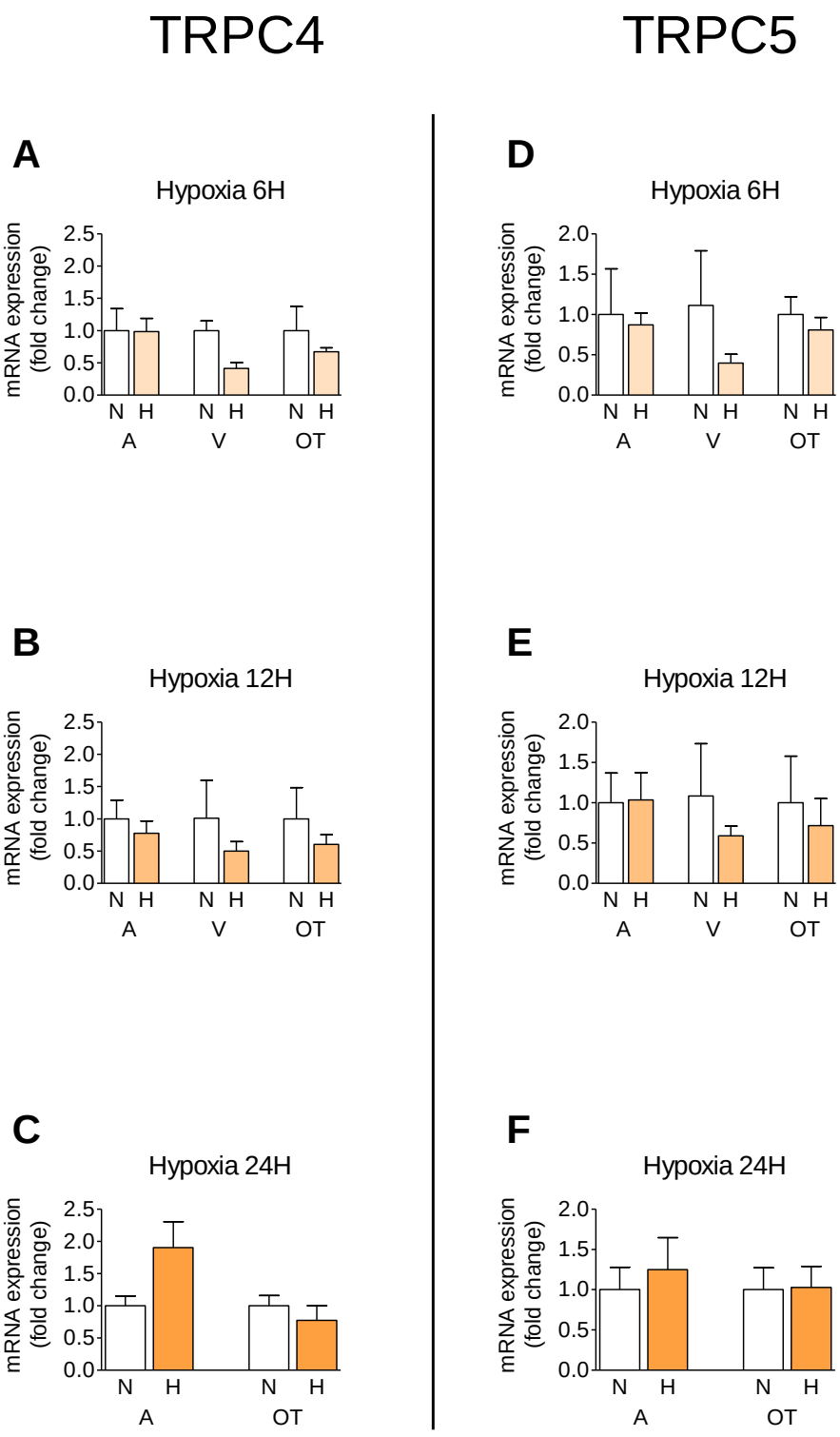

Figure 7. Time-dependent effect of hypoxia (6H, 12H and 24H) on TRPC4 **(A, B, C)** and TRPC5 **(D, E, F)** mRNA expression. The mRNA expression was quantified by RT-PCR performed on isolated atria (A), ventricle (V) and outflow tract (OT) from 4-day-old chick embryo (ED4) exposed to normoxic (N) (control group) and hypoxic conditions (H). TRPC4 n=3-4; TRPC5 n=3-4 determinations.

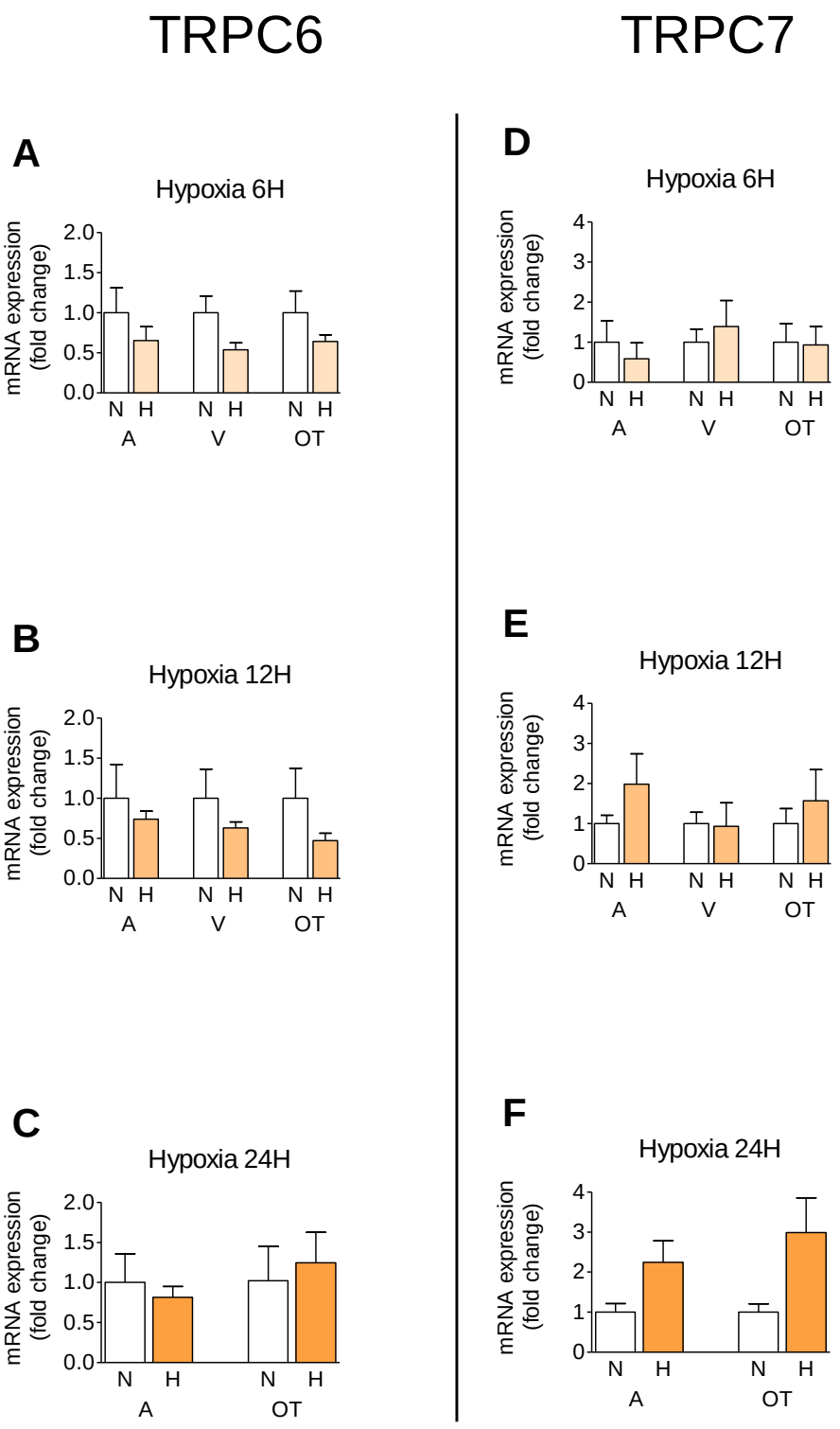

Figure 8. Time-dependent effect of hypoxia (6H, 12H and 24H) on TRPC6 **(A, B, C)** and TRPC7 **(D, E, F)** mRNA expression. The mRNA expression was quantified by RT-PCR performed on isolated atria (A), ventricle (V) and outflow tract (OT) from 4-day-old chick embryo (ED4) exposed to normoxic (N) (control group) and hypoxic conditions (H). TRPC6 n=3-4; TRPC7 n=3-4 determinations.

Distribution of HIF-1α and iNOS mRNA expression in the heart. Both HIF-1α (mean Ct = 25) and iNOS (mean Ct = 27) are well expressed in the 4-day-old chick embryo, as expected at this stage of development. HIF-1α is equally expressed in the atria, ventricle and outflow tract (*Figure 9A*), whereas iNOS mRNA was predominantly expressed in the atria (*Figure 9B*).

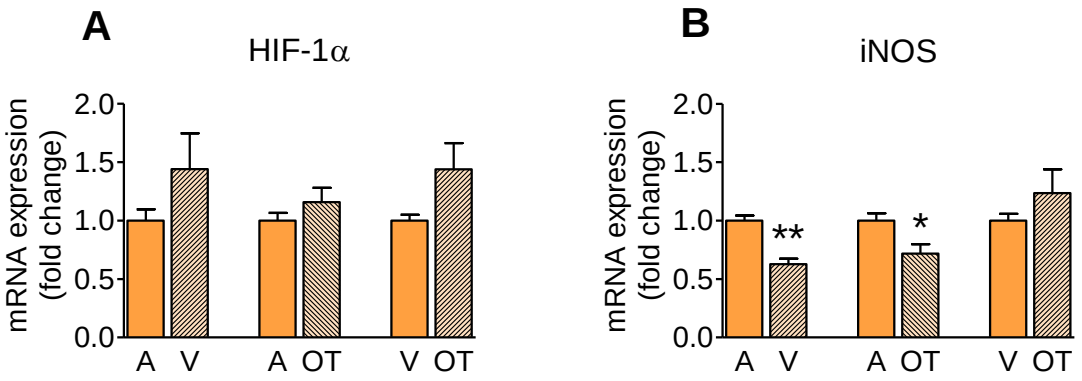

Figure 9. HIF-1α **(A)** and iNOS **(B)** mRNA expression pattern in 4-day-old embryonic chick heart. Quantitative RT-PCR was performed on isolated atria (A), ventricle (V) and outflow tract (OT). iNOS n = 6-7; HIF-1α n=8-14 determinations. **P*< 0.05

Effect of chronic hypoxia on HIF-1α and iNOS mRNA expression. The HIF-1α mRNA expression clearly tends to be down-regulated in the three compartments after 6H, 12H and 24H of hypoxia exposure (*Figure 10A-C*). However, no significant change was observed. On the contrary, the iNOS mRNA expression remains stable after 6H and 12H of hypoxia exposure (*Figure 10D, E*).

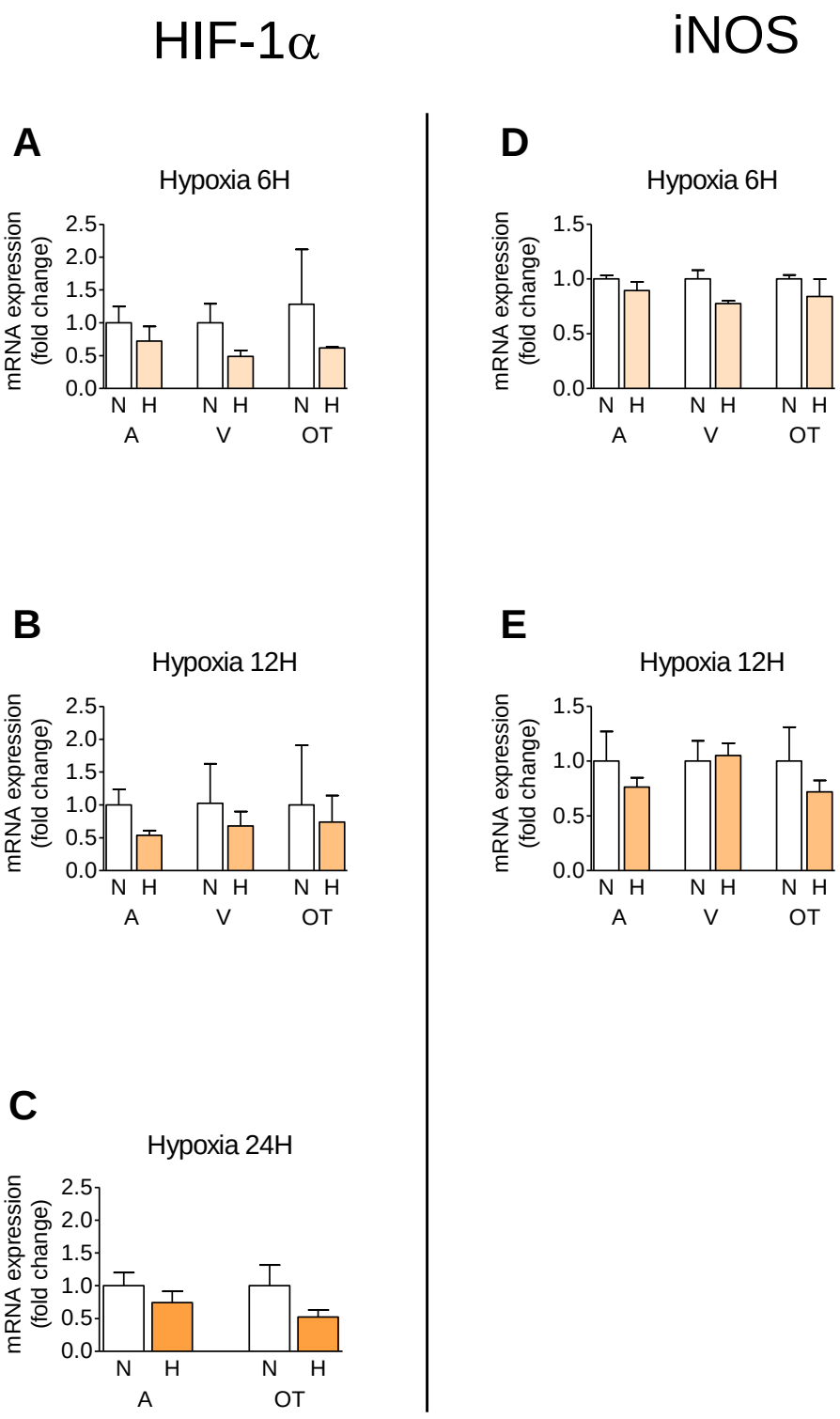

Figure 10. Time-dependent effects of hypoxia (6H, 12H and 24H) on HIF-1α **(A, B, C)** and iNOS **(D, E)** mRNA expression. mRNA expression was quantified by RT-PCR performed on isolated atria (A), ventricle (V) and outflow tract (OT) from 4-day-old chick embryo (ED4) exposed to normoxic (N) (control group) and hypoxic conditions (H). HIF-1α n=3-4; iNOS n=4 determinations.

Discussion

This work assessed for the first time the impact of hypoxia (10% oxygen) on the adenosine receptors and TRPC channels mRNA expression in the developing heart.

Previous experiments in our laboratory have shown the presence of adenosine receptors (40), TRPC channels (57) and iNOS (65) in the embryonic chick heart (ED4) under physiological conditions. The present results confirm the expression of these genes at mRNA level (*Figure 2-4*) and characterize more specifically their expression pattern in the atria, ventricle and outflow tract. Each receptor and channel presents a different expression profile in the heart compartments. The A_1AR is highly expressed in the atria of the embryonic chick heart (ED4). The atria was already pointed as the predominant site of A_1AR expression in the rat embryo (71). Furthermore the A_1AR activation by adenosine plays a central role in the heart rate regulation (34). Hypoxia induces arrhythmias by A_1AR and differently affects atria, ventricle and outflow tract rhythmicity (23). The differential expression might be involved in the higher resistance to hypoxic insult of atria (23). The A_{2a}AR is mainly found in the coronary vessels and is responsible for adenosine-induced vasodilation (72). In the fetal chick, A_{2a}AR so as A_{2b}AR regulate the contractile amplitude of the developing heart (41). A more specific role of these receptors remains unclear. This work shows a predominant expression of A_{2a}AR in the outflow, which might be related to a still unknown specific function in this compartment. The A_3AR activation protects the mature heart from ischemia damage (73). This receptor is equally expressed in the 4-day-old embryonic chick heart, but its function at this stage of development need to be elucidated.

The TRPC channels are known to assembly into homo or hetero-tetrameric structure forming a large variety of functional channels (74). Similarity in the specific compartmental expression of each receptor might be related to possible subunit interaction in the chick heart. In the brain, assembly of TRPC1, TRPC4 and TRPC5, so as TRPC3 and TRPC6 dimerization was identified (75). In our model, similarities are observed between expression of TRPC1-TRPC7, TRPC3-TRPC6 and TRPC4-TRPC5, but dimerization of the subunits was not analyzed.

In order to investigate the impact of chronic hypoxia, the embryos were incubated during 6H, 12H and 24H at 10% oxygen. After 24H of exposure, genetic instability of the internal control, GAPDH, was observed in the ventricle only. Mice embryos at ED11 exposed to chronic hypoxia showed important ventricular morphological changes (20). *In vitro* spontaneously

beating 4-day-old embryonic chick heart exposed to anoxia shows a rapid arrest of ventricular beating activity whereas the atrial activity persists (23). According to this, the ventricle seems to be more sensitive to hypoxic insult than atria.

The adenosinergic system plays a central role in the heart function (34) and is stimulated by hypoxic insult in the early developing embryo (22, 38). Hypoxia significantly increases adenosine level (76, 77). That's why we hypothesize an up-regulation of the adenosine receptors by hypoxia in response to high adenosine level. Our preliminary results show that hypoxia does not significantly modify the receptors mRNA expression. However we observed some tendency over the different hypoxic time exposure, for example the tendency to down-regulation of the A_1AR expression and the up-regulation of A_{2a}AR expression in the ventricle. Otherwise hypoxia has no influence on AR expression. Little is known about the hypoxic regulation of the AR in the embryo. Assays on fetal chromaffin-derived cell line (48) and placental villous explant (49) exposed to chronic hypoxia showed an up-regulation of A_{2a}AR. Studies on endothelial human cells have demonstrated the hypoxic up-regulation of the A_{2a}AR and A_{2b}AR by selective binding of HIF on the HRE of the receptors promoters (78, 79). Further investigations are needed to clarify the AR mRNA regulation in the embryonic heart.

The TRPC channels have a widespread tissue expression (80). Their expression level is tissue specific (81, 82) and depends on the model. Our laboratory has shown the presence of the TRPC1 and TRPC3 to TRPC7 at mRNA and protein level in the embryonic chick heart (ED4) (57) and specified their role in the embryonic heart (57, 58). Our work confirms the mRNA expression and establishes the characteristic spatial expression pattern. The TRPC1 and the TRPC7 channels are equally expressed in the atria, ventricle and outflow tract while the TRPC3 to TRPC6 channels are predominantly expressed in the outflow tract.

TRPC channels inhibition by SKF-96365 provokes significant QTc prolongation which increased the risk of arrhythmias (57). The observed down-regulation tendency of TRPC4, TRPC5 and TRPC6 channels in the ventricle after 6H and 12H of hypoxia might be involved in the arrhythmias induced by the lack of oxygen (23). These preliminary findings, in the 4-day-old embryonic chick heart, show a tendency of down-regulation of the TRPC1 and TRPC6 channels at mRNA level after 6H and 12H of hypoxia. Inconsistent results are found in the literature. In rat and mouse PASMCs, TRPC1 and TRPC6 are involved in the development of hypoxic pulmonary hypertension, and their expression are up-regulated by hypoxia via HIF-1α (59). In 2-day-old rat cardiomyocytes, hypoxia up-regulates TRPC3 and TRPC6 mRNA expression causing cardiac hypertrophy (83). These contradictions might be

due to different models and developmental stages, revealing the difficulty to extrapolate results from a species to another. However supplemental investigations are needed to precisely determine the hypoxic impact on the TRPC channels expression in the embryonic heart.

HIF-1α is a central regulator of the cellular response to hypoxia, involved in many physiological and pathological processes (84). In the poorly oxygenated environment of the embryogenesis, HIF-1α is highly expressed in the 4-day-old chick embryo (stage 24 HH) (8). From stage 25 HH of the developing chick heart, the outflow tract (6) and then the atrioventricular junction and interventricular septum show an important hypoxic level (7). This regional hypoxia triggers HIF-1α expression and works as model for angiogenesis (7). At stage 24 HH we found no differential expression in the heart at mRNA level, HIF-1α was equally expressed in atria, ventricle and outflow tract.

After exposure to 6H, 12H and 24H of chronic hypoxia, we observed in the whole heart a decreased trend of HIF-1α mRNA expression. Based on these preliminary results, recent experiments with quantitative PCR were performed in our laboratory and confirmed with statistical significance the down-regulation of HIF-1α in response to hypoxia in the atria, ventricle and outflow tract. In the epithelial alveolar cells similar down-regulation of the mRNA expression was found after 6H and 12H of hypoxia (85, 86). Despite impact of hypoxia on the mRNA expression, HIF-1α is mainly regulated at post-translational level by protein stabilization (10, 87). Discrepancy can be observed between mRNA and protein expression. In different cell lines, the mRNA down-regulation of HIF-1α was accompanied by an increase of HIF-1α protein (88).

Experiences in the chick, murine and rat embryo have shown that iNOS is highly expressed during embryogenesis (64, 65). An adequate level of NOS and NO production is required for embryonic development (89). In the 4-day-old chick embryo, iNOS is present in the heart with predominant expression in the atria. The presence of iNOS during a particularly hypoxic period of the embryogenesis (65) suggests a hypoxic regulation of the expression. In our model iNOS expression remains stable over the different hypoxic time exposure. Similar lack of hypoxic regulation on iNOS mRNA expression was found in isolated neonatal cardiac cells from rat heart (90). In the mature heart, iNOS is not expressed under physiological normoxic condition, but during hypoxia iNOS mRNA expression is up-regulated by HIF-1α activation (91). Indeed HRE domain is present in the promoter region of iNOS (92).

The protein expression level is not necessarily correlated to mRNA level. The receptors and channels protein level might differ from the mRNA level due to several different regulation steps. As known for HIF-1α, hypoxia might affect the post-translational regulation (10).

In conclusion, the present work confirms the presence of the adenosine receptors, the TRPC channels, HIF-1α and iNOS gene in the 4-day-old embryonic chick heart. In presence of hypoxic environment the AR, TRPC channels, and iNOS mRNA expression remains unaffected. On contrary, the HIF-1α gene is down-regulated by hypoxia. The embryonic gene regulation highly differs from the mature heart response to hypoxia, partly due to different physiological condition and adaptation abilities. This work gives preliminary insight on the pathophysiological response of the embryonic heart to *in ovo* adverse environment.

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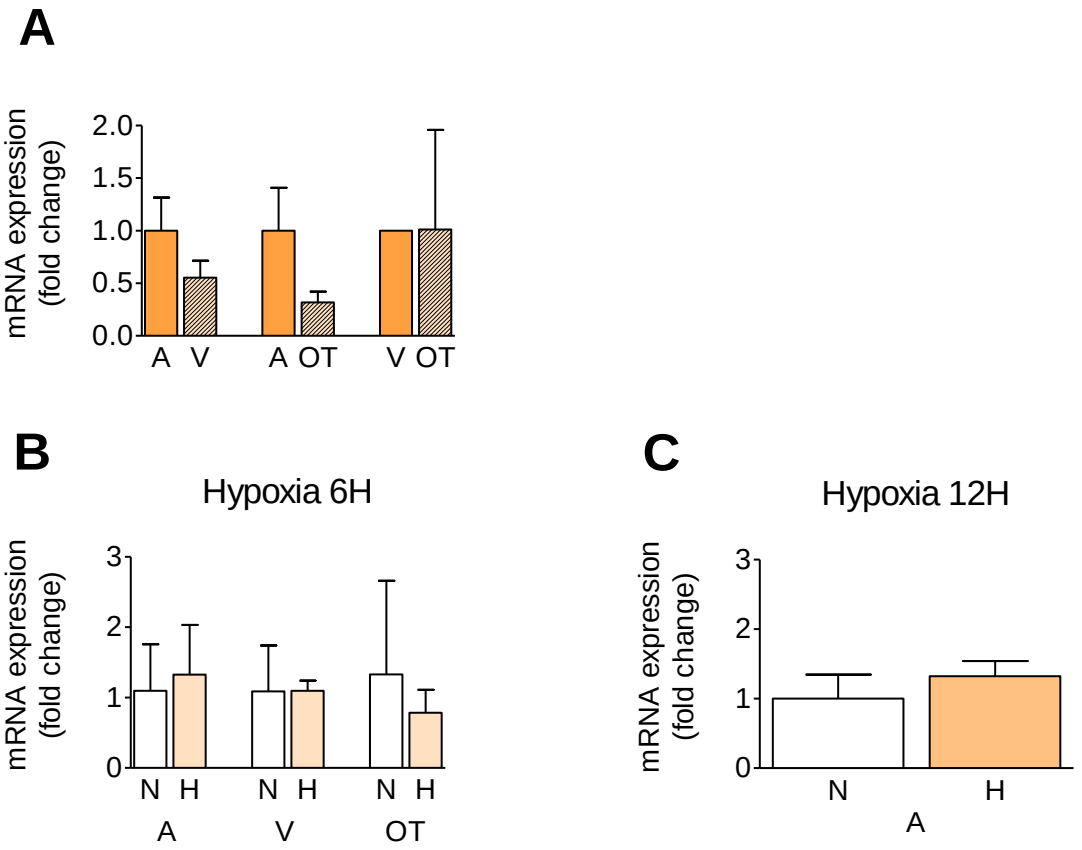
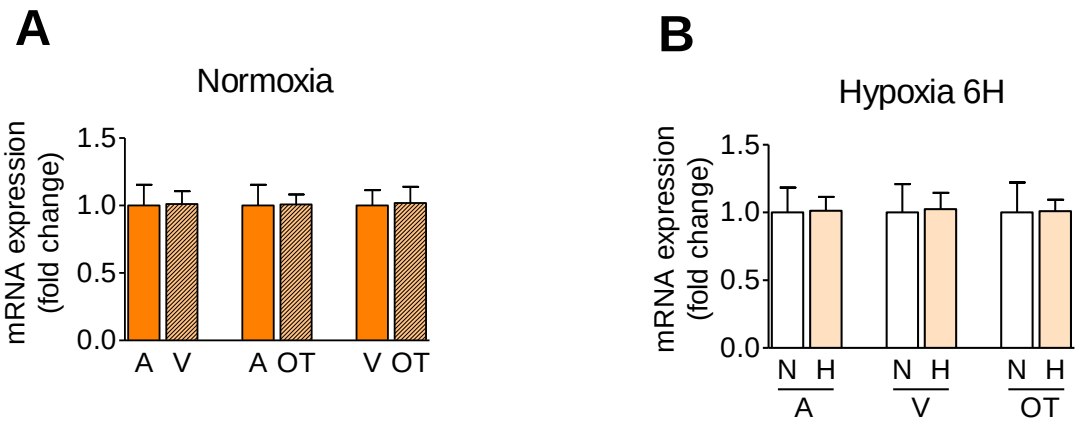
Annexes

	6H			12H			24H		
	A	**V**	**OT**	**A**	**V**	**OT**	**A**	**V**	**OT**
HIF-1α	↓↓≈	↓↓↓	↓↓↓	↓↓↓	↓↓≈	↓↓≈	↓↓↓≈		↓↓↓≈
iNOS	≈≈≈≈	≈≈≈≈	↓↓≈≈	↓↓↑	≈≈≈≈	≈≈↓			
A_1AR	↑↓≈	↓↓≈	↓↓↓	↓↓≈	↓↓↑	↓↓↑	↓↓≈		≈≈↑↓
A_{2a}AR	↑↓≈	↑↑≈	↓↓≈	≈≈≈	↑↑≈	≈≈≈	≈≈↓		↓↓↓≈
A_{2b}AR	↑↓≈	≈≈≈	≈↓						
A_3AR	↑↑↓≈	≈≈↑	≈≈↓	↓↓↑≈	↓↓↓	↓↓≈	≈≈≈≈↓		↑↑↓≈
TRPC1	≈≈≈↓	≈≈↓	↓↓≈	≈≈↓	↓↓≈	↓↓↓	≈≈≈≈≈		↓↓↓≈
TRPC3	≈≈↓	≈≈↑	↑↓≈	↑↑≈	≈≈↓	≈≈↑	≈≈≈≈≈		≈≈≈↑
TRPC4	≈≈↓	↓↓↓	↓↓≈	↓↓≈≈	↓↓≈	↓↓≈	↑↑↑≈		↓↓↓↑
TRPC5	≈≈≈	↓↓↓	≈≈↓	↑↓≈	↓↓≈	↓↓≈	≈≈≈↑		↓↓↑≈
TRPC6	↓↓≈	↓↓↓	≈≈↓	≈≈↓	↓↓≈	↓↓↓	≈≈≈↓		≈≈↓↑
TRPC7	↓↓≈	≈≈↑	↑↓≈	↑↑↓	↓↓↑	↑↓≈	↑↑↑≈		↑↑↑≈

Annexe 1. Summary of hypoxic regulation of the mRNA expression. Each arrow or symbol represents one assay. The arrows do not express significant variation, but clear tendency. ↓ and ↑ for down-regulation and up-regulation, respectively compared to normoxia and ≈ for no change in expression.



Annexe 2. Results of A_{2b}AR mRNA expression. **(A)** mRNA expression pattern of A_{2b}AR in the 4 day-old embryonic chick heart. Time-dependent effect of hypoxia 6H **(B)** and 12H **(C)** on A_{2b}AR mRNA expression. The mRNA expression was quantified by RT-qPCR performed on isolated atria (A), ventricle (V) and outflow tract (OT) from 4-day-old chick embryo (ED4) exposed to normoxic (N) (control group) and hypoxic conditions (H). A_{2b}AR n=2-5 determinations.



Annexe 3. GAPDH mRNA expression. **(A)** mRNA expression pattern of GAPDH in 4-day-old embryonic chick heart (ED4, 24 HH). **(B)** Effect of 6H hypoxia on GAPDH mRNA expression. Quantitative RT-PCR was performed on isolated atria (A), ventricle (V) and outflow tract (OT). GAPDH n=4 determinations.