NOS/sGC/cGK1 pathway in cardiac-specific differentiation of mouse embryonic stem cells

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The influence of nitric oxide synthetase (NOS)/soluble guanylyl cyclase (sGC)/cGMP-dependent protein kinase I (cGKI) pathway in adult cardiac performances is well defined. Indeed, physiological amounts of NO generated by NOS1 and NOS3 or pharmacological administration of NO-donor drugs can influence cardiac contractility and increase coronary blood flow throughout the Ser/Thr phosphorylation (sGC/cGKI pathway) and directly by S-nitrosylation of several proteins.

Although the role of NOS/sGC/cGKI is already extensively studied in adult cardiac cells, the influence of this pathway as trigger/modulator of cardiac differentiation of embryonic stem cells (ESCs) is less defined. Therefore, we decided to investigate the influence of NOS/sGC/cGKI in the early stage of cardiac differentiation of mouse embryonic cells, studying i) enzyme expressions and activities during the cardiac maturation and ii) the acute and chronic effect of pathway alteration.

The mouse embryonic cell line CGR8 was used for these experiments: cells were cultured in undifferentiated form using LIF. Hanging drop methodology was used to promote CGR8 cardiac differentiation. This method allowed cells to aggregate and eventually form embryoid bodies (EBs). The cardiac maturation process was followed for 21 days. Beating EBs were monitored starting from 7^{th}-10^{th} day. At different stages of cardiac maturation, mNos3, mGuCy1b and mPrkgI expressions were detected by real-time PCR (Q-PCR); western blot analysis and enzymatic activity were used for protein evaluation. Cytosolic calcium dynamics was evaluated using epifluorescence microscopy in spontaneous beating EBs from 13^{th}-16^{th} day of differentiation.

Real-time PCR showed that during differentiation the expression of the enzymes increased in a time-dependent mode and different time-courses. The peak of mNos3 expression was measured at day 8 of differentiation and then rapidly decreased. mGuCy1b and mPrkgI were detected starting from day 5 and increased until day 14, maintaining similar values until the end of observation period. Protein expressions were in line with RNA messenger measures, with detectable level of NOS3 only at the beginning of differentiation (days 5 and 8). Enzymatic activities were tested at day 15: sGC activity was increased of 3 times with the NO-donor SNAP and inhibited by 50% with ODQ. Moreover, cyclic GMP was reduced when EBs were incubated for 30 min with L-NMA, suggesting an endogenous activity of NOS. The phosphorylation of Ser/Thr residues on target proteins, were increased by membrane permeable cyclic GMP, isosorbide-5-mononitrate and SNAP and reduced by KT5823, a selective cGKI inhibitor. Functionally, cell incubation with isosorbide-5-mononitrate from the beginning of differentiation slightly increased the percentage of beating EBs at early stages of cardiac maturation (days 5 and 8). In spontaneous beating EBs, acute block of NOS endogenous activity with L-NMA (100 μmol/L) caused a 50% increase of calcium transient frequency. The effect developed progressively during 10 minute exposure and remained constant thereafter. Additionally, a 30-40% reduction of calcium transient amplitude was observed upon NOS blockade, associated with a slight speed of calcium transient decay velocity.

The time-dependent expression of NOS/sGC/cGKI during cardiac differentiation suggests a potential role of these pathways to trigger cardiomyogenesis and modulate calcium handling properties in the developing cardiomyocytes. Further functional data will clarify its role in cardiac maturation.

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