A comparison between human male and female endothelial cells: role of sex in the *in vitro* response to nitric oxide deprivation

M. G. Cattaneo, E. Cappellini, S. Pisoni and L. M. Vicentini

Dept. of Medical Biotechnology and Translational Medicine, Pharmacology Unit, University of Milan, Italy

Loss of normal endothelial function is a critical step in the development and clinical expression of atherosclerosis and cardiovascular disease (CVD). Although CVD has sometimes been considered a disease that predominantly affects men, it is the leading cause of death among both men and women globally. However, there are substantial sex/gender differences in CVD. The prevalence of CVD increases with age in both women and men but is less prevalent in women than men until midlife. Thereafter the gap narrows until the sixth decade, when the prevalence of CVD no longer differs between the sexes. The female advantage in younger women has been attributed to vascular protection by estrogens, which is lost with menopause.

To characterize the sex-dependent differences involved in the establishment of endothelial dysfunction (ED), we have compared male and female endothelial cells (ECs) in an *in vitro* model of ED represented by human umbilical vein ECs (HUVECs) chronically deprived of NO by a 48-h treatment with 5 mM N^G-Nitro-L-Arginine Methyl Ester (L-NAME). Importantly, we always used HUVECs pooled from two or more donors to minimize the variability associated with cells derived from a single male or female newborn donor.

Firstly, we focused our attention on the properties shown by male and female HUVECs grown in standard conditions *i.e.* 199 medium supplemented with FBS. We confirmed that female HUVECs expressed an higher amount of endothelial Nitric Oxide Synthase (eNOS) protein in comparison to male cells, while metabolic activity and basal Reactive Oxygen Species (ROS) content (evaluated by MTS assay and with the fluorescent dye CM-DCFA, respectively) were similar in cells of both sexes. When HUVECs were acutely treated with L-NAME (5 mM for 10 min), we observed in female cells an early and transient rise in cellular ROS. At variance with acute treatment, in cells chronically exposed to L-NAME we found a decrease in cellular ROS content that was more reproducible in female HUVECs. Other data from our laboratory (see poster Cappellini et al) suggest that this loss in ROS represents an adaptive cellular response to NO deprivation mediated through an increased expression and activity of superoxide dismutase-2 (SOD-2) that is in its turn driven by the ROS-dependent nuclear accumulation of Nrf2. It is therefore possible to hypothesize that a prompt response to environmental changes *i.e.* NO deprivation is especially present in female HUVECs, that through the acute formation of ROS may set in motion the Nrf2 accumulation and the subsequent SOD activation. Moreover, the improved protection from NO deficiency shown by female HUVECs was confirmed by the fact that the decrease in eNOS expression that we consistently observed in the chronic absence of NO was significantly lower in female HUVECs than in male cells.

When female cells were cultured in the absence of estrogens *i.e.* phenol red-free 199 medium supplemented with charcoal-stripped FBS, we observed a significant decrease in metabolic activity and basal ROS content in comparison to growth standard conditions. Interestingly, in the absence of estrogens, the cellular ROS content was not further decreased by chronic NO deprivation, and the ability to generate ROS in response to acute L-NAME treatment was lost. Moreover, also the expression of eNOS was decreased in female HUVECs cultured without estrogens.

In conclusion, our results suggest that female ECs are able to counteract *in vitro* NO loss more efficiently than male ECs. The female advantage seems to be lost in the absence of estrogens, thus confirming the crucial role of the hormone in the vascular protection responsible for the lower prevalence of CVD in women until menopause.