

Microvascular effects of corticotropin-releasing hormone in human skin vary in relation to estrogen concentration during the menstrual cycle

V L Clifton, R Crompton, M A Read, P G Gibson¹, R Smith and I M R Wright²

Mothers and Babies Research Center, Hunter Medical Research Institute, Newcastle, New South Wales, Australia

¹Department of Respiratory and Sleep Medicine, Hunter Medical Research Institute, Newcastle, New South Wales, Australia

²Neonatal Intensive Care Unit, Hunter Medical Research Institute, Newcastle, New South Wales, Australia

(Requests for offprints should be addressed to I M R Wright, Neonatal Intensive Care Unit, John Hunter Hospital, Locked Bag #1, Hunter Region Mail Center, Newcastle, New South Wales, 2310, Australia; Email: iwright@mail.newcastle.edu.au)

Abstract

Females have a significantly greater life expectancy than males, which in part may be due to the cardio-protective effects of the female sex hormone, estrogen, on vascular function. However, the sex-specific mechanisms contributing to these differences are complex and not fully understood. Previously we have reported that corticotropin-releasing hormone (CRH) has potent dilator effects in the female skin circulation via mast cell degranulation. Furthermore the dilator response to CRH was more enhanced in females than in age-matched males, suggesting that estrogens may be involved. In this study we examined whether CRH-induced dilation and endothelial cell-dependent dilation in the skin circulation of pre-menopausal females were associated with changes in

estrogen during the menstrual cycle. CRH-induced dilation (1 nM) was enhanced in the presence of high circulating concentrations of estrogen and a positive correlation was identified between CRH-induced dilation and plasma estrogen concentrations. Endothelial cell-dependent dilation was examined using acetylcholine. Acetylcholine-induced dilation (1 nM) was not correlated with circulating concentrations of estrogen. These data suggest the variation in CRH-induced dilation in the skin microvasculature during the menstrual cycle may be due to estrogenic effects on mast cell function and not due to direct changes in endothelial cell function.

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Introduction

Females have a significantly greater life expectancy than males, which in part may be due to the cardio-protective effects of the female sex hormone, estrogen, on vascular function (Sader & Celermajer 2002). However, the sex-specific mechanisms contributing to these differences are complex and not fully understood. The human skin circulation has been used as a non-invasive model for the examination of peripheral vascular function (Kubli *et al.* 2000). Studies in this organ have demonstrated that there are differences in female vascular function in relation to the menstrual cycle and estrogen concentrations (Bartelink *et al.* 1990, Algotsson *et al.* 1995, Gerhardt *et al.* 2000). A number of studies have described reduced peripheral vasoconstriction during the follicular phase (Freedman & Girgis 2000, Chan *et al.* 2001) and enhanced endothelial-dependent vasodilation in the presence of increased estrogen concentrations during the menstrual cycle (Bungum *et al.* 1996, Arora *et al.* 1998) or following long-term

estrogen therapy in post-menopausal women (Arora *et al.* 1998) and in men (New *et al.* 2000). Estrogen may promote vasodilation by increasing endothelial and smooth muscle nitric oxide (NO) production (Forte *et al.* 1998, Chambliss & Shaul 2002) via estrogen receptor alpha (Chen *et al.* 1999, Wyckoff *et al.* 2001). However, evidence collected from our laboratory would suggest there may be an alternate mast cell pathway involved in the regulation of skin vascular tone (Crompton *et al.* 2003) that could also be affected by changing concentrations of estrogen during the menstrual cycle (Clifton *et al.* 2002).

Previously we have reported that corticotropin-releasing hormone (CRH), a 41 amino acid peptide produced predominately in the central nervous system, has potent dilator effects in the female skin circulation when compared with age-matched males (Clifton *et al.* 2002). Human skin is a known target organ for CRH and proopiomelanocortin (POMC) peptides, and localization studies have demonstrated that CRH, its related peptide urocortin, and POMC proteins and genes are expressed in

the basal layer of the epidermis and also in pilosebaceous cells (Kono *et al.* 2001, Slominski *et al.* 2001). This indicates that human skin cells locally produce CRH and POMC peptides and supports the proposal that a stress-response system similar to the hypothalamic–pituitary–adrenal axis may exist in the skin (Slominski *et al.* 2001).

We have identified that CRH-induced dilation in the skin circulation is via mast cell degranulation (Crompton *et al.* 2003). Mast cells are necessary for the development of allergic reactions, often exacerbated by stress and invading pathogens. Mast cells are found in large numbers in the skin (7000–20 000 mast cells/mm²) (Schmolke *et al.* 1994) and are located in the subpapillary region, around blood vessels, lymphatic structures, epithelial appendages and nerves (Eady *et al.* 1979, Wiesner-Menzel *et al.* 1981, Charlesworth 1997), suggesting that mast cells may have multiple roles in the skin, including the control of blood flow. Previous studies indicate that mast cells are responsive to neuropeptides such as substance P, vasoactive intestinal polypeptide and somatostatin (Lowman *et al.* 1988, Benyon 1989) and CRH receptors have been localized on mast cells (Slominski *et al.* 2001), suggesting they would be responsive to CRH and its related peptides. Theoharides *et al.* (1998) and Singh *et al.* (1999) have reported that both CRH and urocortin increased vascular permeability in rat skin via mast cell degranulation.

Mast cell degranulation involves the release of numerous vasoactive molecules, including histamine and NO (Maurer *et al.* 2003). CRH-induced vasodilation in human skin appears to be mediated by mast cell-derived histamine, as we demonstrated that promethazine hydrochloride, a histamine-1 (H₁) receptor antagonist, significantly reduced CRH-mediated effects (Crompton *et al.* 2003). However, the H₁ receptor antagonist did not completely inhibit the CRH-induced dilation (Crompton *et al.* 2003), suggesting that other vasodilator molecules may be involved. This is consistent with a study by Theoharides *et al.* (1998) whereby partial inhibition of the CRH-induced vascular permeability in rat skin was observed in the presence of the H₁ receptor antagonist, diphenhydramine. Our work indicates that CRH-induced dilation in human skin may also be mediated via histamine-2 (H₂) receptors, as the presence of the H₂ receptor antagonist, ranitidine, partially reduced the CRH-induced dilation (Crompton *et al.* 2003). Previous studies support this work as it has been reported that histamine-induced dilation in the skin is mediated via both H₁ and H₂ receptors (Grossmann *et al.* 1999).

A number of studies have also indicated that CRH-induced dilation is mediated via the NO pathway. Clifton *et al.* (1995) reported that CRH was a potent dilator in the human fetal–placental circulation and was mediated via NO and cGMP. Jain *et al.* (1997) demonstrated that CRH caused dilation in pregnant rat mesenteric arteries via NO. NO synthase (NOS) has been localized in mast cells of the human lung (Gaston *et al.* 1994) and the skin vascular

endothelium (Abd-El-Aleem *et al.* 2000) suggesting that both of these sites are potential sources of NO in human skin. In our study the inhibition of NOS using N^G-nitro-L-arginine methyl ester blocked CRH-induced vasodilation in the human skin circulation (Crompton *et al.* 2003). Conversely Theoharides *et al.* (1998) demonstrated that inhibition of NO synthesis potentiated CRH-induced vascular permeability in rat skin. Our work indicates that vasodilator molecules such as histamine and NO released from mast cells may act together to play a role in the CRH-induced vasodilation in human skin (Crompton *et al.* 2003). Histamine-induced dilation is also mediated by NO (Feldman *et al.* 1996), suggesting that endothelial-derived NO may play a role in CRH-induced dilation in human skin. These studies suggest CRH-induced dilation in human skin may be mediated via histamine and NO derived from mast cell degranulation and/or histamine-induced NO production from the vascular endothelium.

Enhanced CRH-induced dilation in pre-menopausal females when compared with age-matched males may be due to the effects of estrogen on mast cell pathways or endothelial–smooth muscle cell pathways. Estrogen receptor β is widely distributed in the epidermis, blood vessels, dermal fibroblasts and hair follicles of the human (Thorn-ton *et al.* 2003), suggesting estrogen may have several different functions in skin, including the regulation of vascular tone during the menstrual cycle. There have been no specific reports of estrogen receptor localization in skin mast cells but this receptor has been localized in human mast cells of the bladder (Pang *et al.* 1995), lung (Zhao *et al.* 2001) and arterioles (Nicovani & Rudolph 2002). Furthermore in rat peritoneal mast cells, 17 β -estradiol activated mast cell histamine and serotonin secretion (Vliagoftis *et al.* 1992), suggesting mast cell degranulation can be directly regulated by this steroid. Based on this information we initiated the present study to examine the association between changes in estrogen concentration during the menstrual cycle and vascular responses to CRH, acetylcholine and heat. This work would provide evidence of whether estrogen is associated with changes in skin vascular tone either via a mast cell pathway as demonstrated by CRH-induced dilation or an endothelial-dependent pathway as demonstrated by acetylcholine- and heat-induced dilation.

Materials and Methods

Experimental subjects

Non-smoking, pre-menopausal females ($n=11$, 21–41 years), with regular menstrual cycles of 28–32 days, who were not using an oral contraceptive, were recruited to the study following guidelines approved by the Hunter Area Health Human Ethics Committee and The University of Newcastle Occupational Health and Safety Committee. The examination of skin microvascular function was

performed on the volar aspect of the forearm. Subjects with any generalized dermatitis or essential hypertension were excluded. Women were tested at days 6, 16 and 26 of the menstrual cycle in consecutive months for CRH and acetylcholine. These intervals were calculated from day 1 of menses for each individual woman and approximately coincide with the follicular, mid-cycle and luteal phases respectively. A plasma sample was obtained on each of these days for the measurement of estrogen and progesterone. Subject weight, height and age were assessed. Subjects refrained from coffee and food for at least 1 h before the investigation.

Drugs

Synthetic human CRH was obtained from Auspep (Melbourne, Australia). Acetylcholine chloride was obtained from the Sigma Chemical Company.

Laser Doppler and iontophoresis

Microvascular laser Doppler is an established method of assessing the function of blood vessels of the peripheral microvasculature (Kubli *et al.* 2000). Low-intensity laser light is reflected from moving blood cells in the skin circulation and thus a measurement of blood flow is obtained. We used a Periflux 5001 Laser Doppler (Perimed AB, Järfälla, Sweden) with one temperature-regulated iontophoresis probe and one temperature-regulated control probe sited on the anterior aspect of the forearm. The PerLont micropharmacology system was used (Perimed). This system is described elsewhere (Hu *et al.* 1998). Briefly, a transdermal current is applied to cause migration of drugs from a disposable electrode, surrounding the temperature-controlled laser Doppler transducer, into the skin. Blood flow readings are expressed as arbitrary perfusion units (PUs).

Experimental protocol

Subjects were placed in a semi-supine position and skin basal blood flow was recorded for 5 min, after which six doses of CRH (1 nM), acetylcholine (1 nM) or vehicle controls (distilled water) were administered to the skin circulation, on separate occasions, by iontophoresis at a current of 0.06 mA for 30 s per dose with a positive polarity. Blood flow was recorded by laser Doppler after each dose. CRH and acetylcholine were measured separately in consecutive months on days 6, 16 and 26. Consistency of the data collected on individuals who received both CRH and acetylcholine treatments was ensured by comparing the blood flow response to heat between each treatment day or each month. There was no significant difference in response to heat between months (data not shown).

As described previously (Hu *et al.* 1998), certain standard provocations were performed to allow for comparison between different studies and subjects. After the last iontophoretic stimulation and when skin perfusion had returned to a stable level, a blood pressure cuff was used to examine a short period of absent blood flow and the re-perfusion capacity of the circulation in the control probe. This allowed for a biological zero to be obtained in each experiment. Forearm blood flow was then allowed to stabilize before a standard thermal provocation was then used. A small heater around the control probe increased the temperature setting from 40 to 44 °C in 1 ° increments at 60 s intervals. The reactive hyperemia following the heat provocation was monitored by the laser Doppler. At the time of the iontophoresis procedure, a blood sample was obtained from each subject for steroid hormone measurement.

Measurement of estrogen and progesterone

Serum 17 β -estradiol and progesterone levels were measured at day 6, 16 and 26 of the menstrual cycle using commercial RIA kits (Immulite 2000; Diagnostic Products Corp., Los Angeles, CA, USA).

Data analysis

Dose-response curves were compared using generalized estimating equations (GEEs) using the statistical software, STATA (version 7 2001; STATA Press, College Station, TX, USA). Pearson's correlation was used for comparison of age, weight and height with responses to CRH and acetylcholine (Graphpad InStat, Graphpad Software 1990–1993, Version 2.04a). All values were expressed as means \pm S.E.M. unless otherwise stated. $P < 0.05$ was considered significant.

Results

The mean age of women in this study was 26.9 ± 2.1 years. The mean weight of the subjects was 63.0 ± 2.8 kg, mean height 163.3 ± 2.2 cm and mean body mass index 23.6 . The mean length of the menstrual cycle was 28.5 ± 1.2 days. Some women had longer menstrual cycles than others, with their highest concentration of 17 β -estradiol occurring on day 26 rather than day 16 (Fig. 1). Consequently we used the highest estradiol value for each subject, at either day 16 or 26, and then compared the results with 17 β -estradiol levels on day 6 of the cycle. Accordingly the data were analyzed based on low and high estrogen concentrations.

The mean serum concentrations of low 17 β -estradiol was 131.8 ± 8.4 pM (day 6) and high 17 β -estradiol was

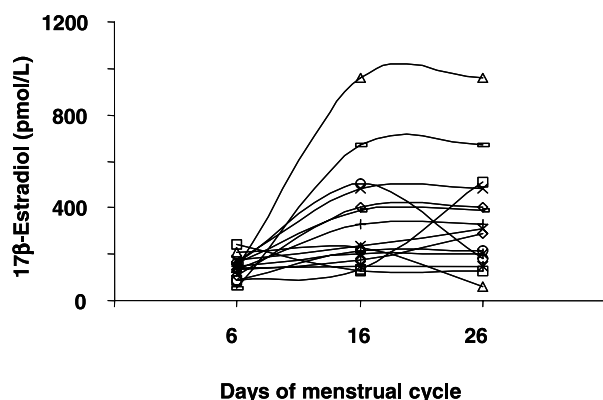


Figure 1 Comparison of individual 17 β -estradiol concentrations on days 6, 16 and 26 of the menstrual cycle. Pre-menopausal women ($n=11$) had variable 17 β -estradiol concentrations during their menstrual cycle, with some women having the highest concentration at day 26.

579.5 ± 154.5 pM (day 16–26). Serum progesterone levels corresponding to low and high estrogen levels were 1.0 ± 0.1 and 21.9 ± 4.8 nM respectively.

Biological zero, basal skin microvascular flow and post-occlusive re-perfusion were not significantly different in the presence of high and low serum 17 β -estradiol concentrations (paired t -test $P>0.05$). Heat-induced hyperemia was significantly enhanced in the presence of high circulating concentrations of estrogen (GEE, $P<0.05$) (Fig. 2).

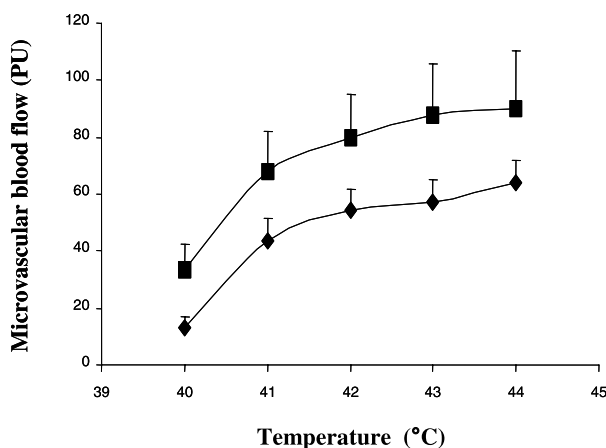


Figure 2 Comparison of heat-induced vasodilation in female skin circulation during the menstrual cycle. Heat-induced dilation was significantly increased in the presence of high circulating concentrations of 17 β -estradiol ($n=11$, ■, day 16–26) when compared with low circulating concentrations of 17 β -estradiol ($n=11$, ◆, day 6) (GEE, $*P\leq 0.001$) during the menstrual cycle. Microvascular skin blood flow was measured in PUs as a small heater around the head of the control probe increased the temperature setting from 40 to 44 °C in 1 ° increments at 60 s intervals. The reactive hyperemia following the heat provocation was monitored by the laser Doppler. All values are expressed as means \pm S.E.M.

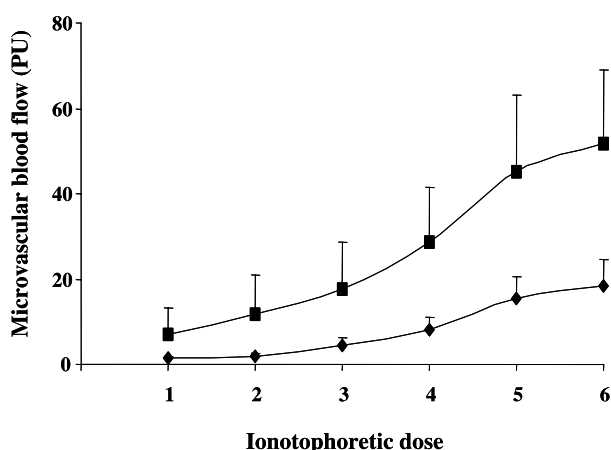


Figure 3 Comparison of CRH-induced vasodilation in female skin circulation during the menstrual cycle. CRH-induced dilation was significantly increased in the presence of high circulating concentrations of 17 β -estradiol (1 nM, $n=11$, ■, day 16–26) when compared with CRH-induced dilation in the presence of low circulating concentrations of 17 β -estradiol (1 nM, $n=11$, ◆, day 6) (GEE, $*P\leq 0.001$) during the menstrual cycle. Microvascular skin blood flow was measured in PUs, and CRH was administered by six 30 s pulses of a positive electrical current at 0.06 mA. All values are expressed as means \pm S.E.M.

Human CRH (1 nM) caused a dose-dependent vasodilation in the skin microvascular circulation (Fig. 3). CRH-induced dilation was significantly greater in the presence of high circulating concentrations of estrogen when compared with low concentrations of estrogen ($n=11$, GEE, $P<0.05$) (Fig. 3). There was no correlation between the CRH-induced dilation and subject age, weight or height.

There was a positive correlation between estrogen concentrations and the effect of CRH on blood flow ($n=11$, $R^2=0.53$, $P<0.0001$) and no correlation between progesterone concentrations and CRH ($n=11$, $R^2=0.01$, $P=0.523$).

Acetylcholine (1 nM) caused a dose-dependent dilation in female subjects ($n=6$) and these responses were not significantly different in the presence of low and high estrogen concentrations (Fig. 4) (GEE, $P>0.05$). There was no correlation between estrogen ($n=6$, $R^2=0.0006$, $P=0.92$) or progesterone ($n=6$, $R^2=0.03$, $P=0.51$) concentrations and maximal dilator response to acetylcholine throughout the menstrual cycle. There was no correlation between response to acetylcholine and subject age, weight or height.

Discussion

We have previously reported that CRH-induced dilation in the skin microvasculature was more enhanced in

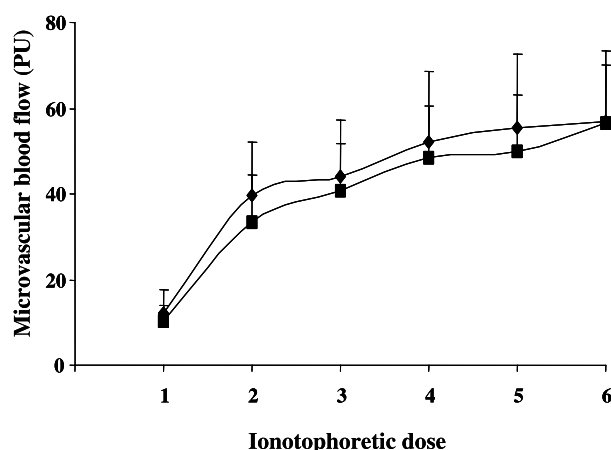


Figure 4 Comparison of acetylcholine-induced vasodilation in female skin circulation during the menstrual cycle. Acetylcholine-induced dilation was not significantly altered in the presence of high (1 nM, $n=6$, ■, day 16–26) and low (1 nM, $n=6$, ♦, day 6) circulating concentrations of 17 β -estradiol (GEE, $P>0.05$) during the menstrual cycle. Microvascular skin blood flow was measured in PUs, and acetylcholine was administered by six 30 s pulses of a positive electrical current at 0.06 mA. All values are expressed as means \pm S.E.M.

pre-menopausal females when compared with age-matched males (Clifton *et al.* 2002) and that CRH-induced vasodilation occurred primarily via mast cell degranulation and was partially mediated by histamine, prostaglandins and NO (Crompton *et al.* 2003). In our present investigation we have determined that CRH-induced dilation in the skin microvasculature of pre-menopausal females is positively correlated with circulating concentrations of endogenous estrogen. However, there was no correlation between estrogen concentrations and endothelial-induced dilation with acetylcholine. These data suggest that changes in skin microvascular function in response to CRH may be linked to alterations in mast cell function rather than direct changes to endothelial function during the menstrual cycle.

Variations of female cardiovascular function during the menstrual cycle are known to be associated with changes in circulating estrogen levels (Hashimoto *et al.* 1995, Kawano *et al.* 1996, Giannattasio *et al.* 1999, Gerhardt *et al.* 2000). Arora *et al.* (1998), using iontophoresis and laser Doppler, demonstrated that skin microvascular dilator responses to acetylcholine and sodium nitroprusside were enhanced in mid-cycle pre-menopausal females and post-menopausal females receiving estrogen replacement therapy, when compared with post-menopausal females who had not received estrogen supplements. Williams *et al.* (2001) examined acetylcholine- and sodium nitroprusside-induced dilation in the skin microvasculature of 15 pre-menopausal females at four time points during the menstrual cycle and reported an enhanced response to acetylcholine in the late follicular phase.

However, these changes did not appear to be correlated with the changes in circulating estrogen levels (Williams *et al.* 2001). In agreement with these findings, our study has similarly demonstrated that acetylcholine-induced dilation is not correlated with circulating concentrations of estrogen during the menstrual cycle. We did not find an enhanced response to acetylcholine in the follicular phase as reported by Williams *et al.* (2001). The discrepancy between the two studies may be due to methodological differences as we used fewer subjects, a lower concentration of acetylcholine and measured blood flow directly from the chart recording after multiple administrations of the drug, while Williams *et al.* (2001) administered the drug once and then quantified the change in blood flow by measuring the area under the curve. Based on these differences between the studies these findings need to be investigated further.

In the coronary circulation, acute administration of estrogen to post-menopausal women enhanced acetylcholine-induced dilation (Gilligan *et al.* 1994, Pinto *et al.* 1997). Flow-mediated dilation in the brachial artery was enhanced in pre-menopausal women due to increased endothelial NO production and was associated with changes in circulating concentrations of estrogen (Hashimoto *et al.* 1995, Sudhir *et al.* 1996). Majmudar *et al.* (2000) examined vascular responses to NO inhibition in the brachial artery of pre-menopausal females and concluded enhanced vasodilation was due to increased endothelial NO production when compared with post-menopausal females and males. Furthermore, long-term estrogen replacement therapy in post-menopausal women restored endothelial NO production to levels similar to those observed in pre-menopausal women (Majmudar *et al.* 2000). Since endothelial-dependent vasodilation using acetylcholine did not change with increased circulating estrogen levels in our study, we cautiously conclude that other factors regulated by estrogen, such as mast cell pathways, may play a role in modulating vascular tone in the skin during the menstrual cycle.

Estrogens are known to modulate mast cell function in the human. Kim *et al.* (2001) reported that a human clonal mast cell line, HMC-1, reduced its release of pro-inflammatory cytokines following incubation with estrogen *in vitro*. IgE-mediated histamine release from rat peritoneal mast cells and human basophils *in vitro* is enhanced following incubation with estrogen (Cocchiara *et al.* 1990). Mast cell degranulation may be increased during the menstrual cycle, as demonstrated by the correlation of urinary histamine metabolites and plasma estrogen concentrations in pre-menopausal women (Jonassen *et al.* 1976). Furthermore, responses to histamine in skin prick tests and in the nasal mucosa of atopic and non-atopic pre-menopausal women are greatest at mid-cycle (Kalogeromitros *et al.* 1995, Haeggstrom *et al.* 2000). From our study, the enhanced response to CRH-induced dilation in association with increased circulating estrogen

concentrations may be caused by increased mast cell degranulation in female skin.

There are several different vasoactive substances released by mast cells that may affect vascular tone (Boesiger *et al.* 1998, Kim *et al.* 2001), with histamine being the most widely measured factor (Maurer *et al.* 2003). Our research indicates histamine, prostaglandins and NO may mediate CRH-induced dilation in the skin (Crompton *et al.* 2003). When we examined histamine-induced dilation in the skin microvasculature of premenopausal women at mid-cycle and compared the response with that in males, we found there was no significant difference between the sexes (Crompton *et al.* 2003) even though there was a significant difference in CRH-induced dilation between the sexes (Clifton *et al.* 2002). Furthermore, when we examined endothelium-dependent dilation using acetylcholine between age-matched males and females we also observed no significant difference in the response (Clifton *et al.* 2002). It was concluded from this work that CRH-induced dilation in the skin microvasculature may be mediated by histamine and NO, but these factors alone did not account for the difference in response to CRH between males and females (Clifton *et al.* 2002). These data suggest that alterations in the skin CRH–mast cell pathway may be influenced by the different phases of the menstrual cycle.

CRH and urocortin act via the CRH receptor isoforms, type 1 and 2 (Aguilera *et al.* 2004). In human skin, the type 1 α isoform is expressed in most cell types of the epidermis and dermis (Slominski *et al.* 2004). The type 2 isoform is found in hair follicles, sebaceous glands and blood vessels (Slominski *et al.* 2004). CRH and urocortin may act via the type 1 CRH receptor on skin mast cells, as the type 1 receptor mediates cardiac mast cell degranulation in the rat (Pang *et al.* 1998). Our data suggest that the response to CRH in the skin microvascular circulation is mediated via a CRH receptor, as we were able to suppress CRH-induced dilation by the administration of a CRH antagonist, α -helical CRH_(9–41) (Clifton *et al.* 2002). Previous studies in the human placental circulation suggest that CRH-induced dilation is mediated by a type 2 CRH receptor (Leitch *et al.* 1998) and the NO pathway (Clifton *et al.* 1995). It is possible that CRH-induced dilation in the skin could be mediated by CRH receptors 1 and 2 present on mast cells and the endothelium respectively.

We have observed an increased skin vasodilator response to hyperemia with high circulating concentrations of estrogen. Other studies have shown that the skin response to heat is more enhanced in pre-menopausal females than males (Bartelink *et al.* 1993). High concentrations of combination oral contraceptives, ethinyl estradiol and progestin, in pre-menopausal women are associated with an enhanced vasodilator response to increased skin temperature when compared with low concentrations of these hormones (Charkoudian &

Johnson 1999a). These data suggest that female sex steroids influence the thermal control of skin blood flow. The vasodilator response to increased skin temperature is partially mediated by NO (Kellogg *et al.* 1998, Shastry *et al.* 2000), but not via acetylcholine muscarinic receptors (Shastry *et al.* 2000) or prostaglandins (Charkoudian & Johnson 1999b). Since there was no association between endothelial-induced dilation and estrogen in our study, it is possible that the changes in the vasodilator response to heat may be due to estrogen effects on the neuromuscular or endocrine cells of the skin.

This study indicates that circulating estrogen concentrations influence CRH-induced dilation in the female skin microcirculation. Interestingly, acetylcholine-induced dilation did not vary with estrogen concentrations during the menstrual cycle. However, this finding requires further investigation and may only be relevant to the skin circulation. These data, and in combination with our previous studies (Clifton *et al.* 2002, Crompton *et al.* 2003), suggest that the variation in CRH-induced dilation during the menstrual cycle is due to alterations in mast cell function. These studies highlight that some components of the immune system have a role in the control of skin vascular function and may be a contributing mechanism to the cardio-protective effects of estrogen.

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