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Endothelial Dysfunction Does Not Occur after Acute, Elevated Homocysteine Exposure of the Lumen of the Iliac Artery of the Anaesthetised Pig

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Keywords
Homocysteine · Endothelium · Vascular disease · Shear stress

Abstract
Introduction: Elevated luminal homocysteine has been linked with cardiovascular disease; however, whether there is a direct effect of homocysteine on blood vessel endothelium is not clear. In this study, the acute effect of luminal homocysteine on iliac artery endothelial function was assessed in the anaesthetised pig. ***Methods:*** Hyperhomocysteinaemic blood was injected into an occluded segment of the iliac in the anaesthetised pig for 20 min, and the effect on atrial diameter during the occlusion and during the reactive hyperaemia assessed. ***Results:*** No significant changes in arterial diameter or pressure were observed during the incubation period at homocysteine concentrations of 10, 20, 40 and 100 μM. There was also no difference in the magnitude of the iliac diameter increase in the response to reactive hyperaemia when the incubation period was completed. ***Conclusion:*** There is no evidence of endothelial dysfunction in response to an acute 20-min elevation in homocysteine in an intact conduit artery.
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Introduction

There is evidence that the association between homocysteine and cardiovascular disease is causal [1]. Human vascular endothelial cells are damaged by homocysteine concentrations and the damage is thought to be mediated by H_2O_2 [2, 3] homocysteine also activates a number of pro-thrombotic pathways [4]. An in vitro study on human umbilical vein endothelial cells showed that homocysteine reduced both intracellular and culture medium nitric oxide (NO) levels and decreased phosphorylation of endothelial nitric oxide synthase. In addition, homocysteine also reduced vascular endothelial growth factor expression in a dose-dependent manner [5]. A predisposition to arterial disease(s) has been reported in the presence of elevated concentrations of homocysteine [6–12] so much so that it has been suggested that hyperhomocysteinaemia is predictive of future repeat acute coronary syndromes [13]. However, how rapidly high luminal concentrations of homocysteine can exert a direct negative effect on arterial function in vivo is unknown. Therefore, the aim of the present study was to evaluate in vivo the acute (20-min) effect of direct exposure of conduit arterial endothelium to elevated concentrations of homocysteine on arterial endothelial function, as indicated by changes in tone and in shear stress-mediated dilation.

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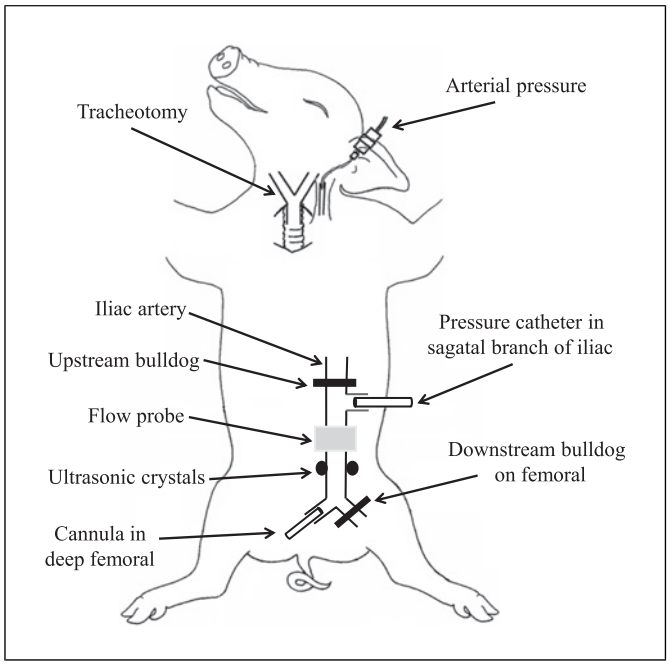


Fig. 1. Schematic diagram of the experimental set-up.

Methods

General Surgery

Eight female landrace pigs (20–25 kg) were sedated with ketamine (14 mg/kg) and xylazine (2.7 mg/kg) and then anaesthetised with a bolus, followed by a continuous infusion, of sodium pentobarbital (induction 30 mg/kg i.v.; maintenance 6 mg/kg/h i.v.). End-tidal carbon dioxide partial pressure ($PETCO_2$) was measured continuously with a capnograph (SurgiVet, Smiths Medical, Dublin, OH, USA). Arterial pH, PCO_2, and PO_2 were assessed using an i-STAT blood gas analyser (Abbot Point of Care Inc., Princeton, NJ, USA) and maintained within their normal ranges. Following tracheotomy the lungs were ventilated with a Palmer pump at a ventilation rate of 20 per minute at a tidal volume adjusted to keep end-tidal and arterial PCO_2 normal; 40% O_2 in room air, to which we added oxygen sufficient to maintain arterial blood fully saturated.

Iliac Artery Instrumentation

Figure 1 shows a schematic of the experimental preparation of the iliac artery, the aim was to create an isolated segment of the iliac to assess the effect of luminal exposure to homocysteine. The pigs were prepared as previously described [14]. Briefly, a cannula attached to a pressure transducer (Grass, Grass Technologies, West Warwick, RI, USA) was inserted into the left carotid artery for arterial blood pressure measurement. Either the left or right iliac was instrumented, blood flow was measured by placing an ultrasonic transit time flow transducer around the iliac (Transonic Systems Inc, Ithaca, NY, USA), iliac diameter was measured using piezoelectric crystals connected to a sonomicrometer (Somometrics Corporation, London, ON, Canada), pressure was measured by placing a catheter tipped manometer (6F, Millar, Houston, TX, USA) into the sagittal branch of the iliac, a cannula was inserted into the deep femoral artery to allow the injection of control or homocysteine blood, when required a proximal occluder (upstream bulldog clip) was placed above the site of pressure and iliac flow were measured, and a distal occluder (downstream bulldog clip) was placed on the femoral downstream of the deep femoral cannula. All parameters were recorded using Power lab preamplifiers and software (AD Instruments Ltd, Oxford, UK), and a Dell PC. At the end of the experiments, animals were killed using a lethal intravenous injection of KCl and pentobarbitone.

Experimental Protocol

Once the iliac instrumentation was complete the following procedure was followed in the same sequence.

1) First a control response to normal blood isolated in the test segment was established. A bulldog clip was applied upstream of the pressure and diameter measurement site. This stopped blood flow and effectively drained the iliac of blood, as can be seen by the fall in iliac diameter in Figure 2.
2) The downstream bulldog clip was placed on the femoral to create an isolated segment of the iliac.
3) Control heparinised blood was then injected into the now isolated iliac segment via the deep femoral artery until the diameter reached the artery diameter before application of the upstream bulldog clip.
4) This was maintained for 20 min.
5) At the end of 20 min both bulldog clips were removed and flow re-established and the effect of occlusion on the ability of the iliac test segment to dilate in response to blood flow was assessed.
6) Artery diameter and blood flow were allowed to return to baseline before the application of homocysteine.
7) Once baseline diameter and blood flow occurred, the procedure was repeated by injecting blood containing homocysteine 10 μM into the test segment for 20 min.

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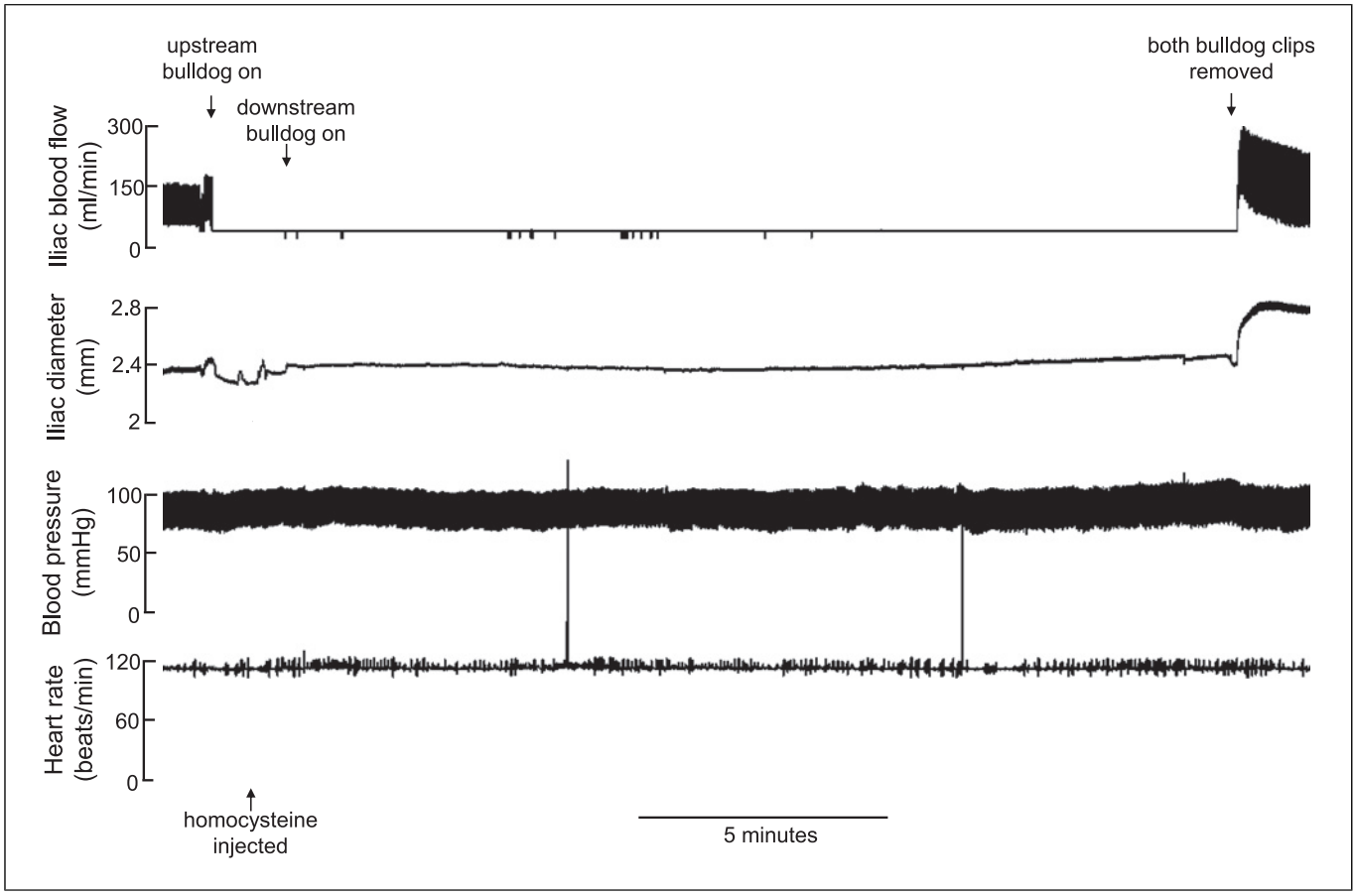


Fig. 2. An example of the records taken in one experiment (pig 30/11), showing the response of the iliac artery to homocysteine 40 μM over a 20 min period. Note the slight increase in diameter during this cessation of blood flow following application of the bulldog clips for the 20 min incubation period and the rapid increase in blood flow when removed (reactive hyperaemia) and the iliac diameter increase in response.

8) As the artery segment response was unaffected by this initial concentration, the procedure was repeated after parameters returned to baseline using blood concentrations of 20 μM and subsequently 40 μM.
9) In four pigs a higher concentration of blood containing 100 μM homocysteine was also used.
10) One control exposure was conducted in every animal in addition to several subsequent interventions using homocysteine-containing blood, the number of interventions conducted in each pig are listed in the rows of the Tables 1 and 2.

Shear stress- and NO-mediated artery dilatation (flow-mediated dilatation) was assessed, caused by the increased blood flow of reactive hyperaemia upon release of occlusion of the artery, an example records is shown in Figure 2. Diameter changes are reported as the difference between the diameter at the end of occlusion from the diameter at the beginning of occlusion.

Statistical Analysis

Non-parametric Mann-Whitney U test was used to compare data (SPSS) due to the low number of animals used. Also, the lack of effect observed did not justify using more pigs. A $p < 0.05$ was taken to indicate significance.

Results

Figure 2 is an example of the records obtained from one experiment (pig 30/11), showing the response of the iliac artery to homocysteine 40 μM. The diameter of the iliac in this experiment increases slightly during the 20-min incubation with homocysteine. Flow-mediated dilation of the iliac is evident following the removal of the bulldog clips and the resumption of normal unimpeded blood flow. Tables 1 and 2 summarise the data obtained from all pigs used and clearly show no significant effect of

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Table 1. Group data for the effect of homocysteine (10, 20, 40 and 100 μM) on change in iliac artery test segment diameter over the 20 min incubation period which shows no significant effect of homocysteine, at each concentration, compared to the controls (n = 8)

Pig No.	Control	Homocysteine, μM			
		10	20	40	100
30/11	−0.03	0.09	0.01	0.10	
31/11	0.35	0.16	0.21	0.01	
32/11	−0.07	−0.17	0.05	−0.04	
33/11	−0.01	0.04	−0.04	−0.18	
34/11	−0.03	0.11	0.00	0.04	0.05
39/11	0.05				0.02
40/11	−0.55				−0.01
41/11	0.02				0.16
Mean	−0.03	0.04	0.04	−0.01	0.06
SD	0.25	0.13	0.10	0.11	0.08
n	8	5	5	5	4
SEM	0.09	0.06	0.04	0.05	0.04
p value		0.24	0.24	0.88	0.20

Table 2. Group data for the effect of homocysteine (10, 20, 40, and 100 μM) on the ability of the iliac artery to dilate in response to the increase in blood flow during reactive hyperaemia 20 min incubation

Pig No.	Control	Homocysteine, μM			
		10	20	40	100
30/11	1.18	0.83	0.86	1.14	
31/11	1.15	0.65	0.52	0.78	
32/11	0.94	0.53	0.42	0.58	
33/11	0.48	0.38	0.24	0.45	
34/11	0.42	0.24	0.20	0.41	0.85
39/11	0.30				0.62
40/11	0.23				0.48
41/11	−0.01				0.39
Mean	0.59	0.53	0.45	0.67	0.59
SD	0.45	0.23	0.26	0.30	0.20
n	8	5	5	5	4
SEM	0.16	0.10	0.12	0.13	0.10
p value		1.00	0.61	0.66	0.80

There was no significant difference between the effect of homocysteine at any concentration on flow-mediated dilation due to the reactive hyperaemia (n = 8).

homocysteine, when compared to control blood, on the change in iliac diameter during the 20 min incubation of homocysteine (Table 1) or the flow-induced increase in diameter as a result of the hyperaemia (Table 2).

Discussion

The results show that 20 min exposure of the conduit artery to concentrations of homocysteine, known to be associated with recurrence of acute coronary syndromes, did not cause significant endothelial dysfunction [13] since the iliac dilated in response to the increase in blood flow following removal of the bulldog occluders. Dilation is known to be dependent on the presence of a functional, intact endothelium in the iliac in vivo [15]. This indicates that homocysteine does not immediately interact with any factors that control minute-by-minute changes in arterial tone, and does not immediately interfere with glycocalyx function or the endothelial production of NO in conduit vessels in response to changes of shear stress, as specifically evidenced by the lack of diameter change differences between the control and homocysteine experiments and the maintained reactive hyperaemia [16].

The lack of an effect may be due to the relatively short duration of exposure since the mechanism usually proffered for endothelial dysfunction and damage caused by homocysteine involves the production of reactive oxygen species production of which may take a lot longer than the 20 min exposure time in our experiments [3]. The concentrations used in the present study were initially within the mildly elevated range (above 12 μM) predictive for the recurrence of acute coronary syndromes [13], and which caused endothelial dysfunction in aortic rings from rats fed excess methionine to produce moderate hyperhomocysteinaemia [5].

It should be noted that although homocystinuria is associated with aggressive premature arterial disease, some other genetic causes of more moderate hyperhomocysteinaemia are not [17]. This leads to the possibility that the arterial disease associated with hyperhomocysteinaemia is not due to the hyperhomocysteinaemia per se but to an interaction between hyperhomocysteinaemia and other atherothrombotic risk factors. Some doubt has been cast on the hypothesis that hyperhomocysteinaemia causes arterial disease because

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the normalisation of homocysteine blood concentrations with vitamins like folic acid does not appear to have any cardiovascular benefit [1]. To use such evidence to deny the importance of screening patients for hyperhomocysteinaemia is illogical; such patients still need to be cared for as high risk. Moreover, such treatment failures so far do not invalidate the evidence for homocysteine as a cause of, and a high risk for, arterial disease but may indicate that vitamin treatment of patients with hyperhomocysteinaemia with this particular risk factor is not effective and that other measures, possibly pharmacological, need to be explored [1].

Limitations

The primary limitation of the present study was the short duration of luminal exposure to homocysteine, since in cardiovascular disease the hyperhomocysteinaemia would likely persist for a considerable period of time. However, it is still worthwhile to explore whether acute exposure to elevated arterial luminal levels of homocysteine in a large animal model has an immediate effect on endothelial function. In view of the absence of a direct acute local exposure of artery to hyperhomocysteinaemia in the present study, we conclude that the mechanism of homocysteine-related arterial disease is the result of more prolonged exposure, and or due to a metabolic effect of systemic hyperhomocysteinaemia.

Statement of Ethics

This investigation was carried out under licenses (ref no: B100/2838) issued by the Department of Health Ireland as directed by the Cruelty to Animals Act Ireland and EU Statutory instructions.

Conflict of Interest Statement

The authors have no conflicts to declare.

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Author Contributions

Farouk Markos, Andrew J. O'Leary, Mark I.M. Noble, and Therese Ruane-O'Hora conducted the experiments, analysis, and writing of the manuscript.

Data Availability Statement

All data generated or analysed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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