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Source: PFLUGERS ARCHIV-EUROPEAN JOURNAL OF PHYSIOLOGY
Volume: 456 Issue: 3 Pages: 635-645 Published: JUN
2008

A novel method of measuring nitric oxide dependent fluorescence using 4,5-diaminofluorescein (DAF-2) in the isolated Langendorff perfused rabbit heart

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Running title: NO fluorescence in isolated hearts

Keywords: nitric oxide, isolated hearts, fluorescence, DAF-2

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Abstract

4,5-diaminofluorescein (DAF-2) has been used to measure nitric oxide (NO) activity from a variety of preparations. The aim of this study was to develop a method to assess changes in NO fluorescence using DAF-2 in isolated rabbit hearts (2.0-2.5kg;n=8). Hearts were perfused in constant flow Langendorff mode and instrumented to record aortic perfusion pressure, left ventricular pressure and left ventricular epicardial fluorescence using a bifurcated light guide at excitation wavelengths of $470\pm 10\text{nm}$, $480\pm 10\text{nm}$, $490\pm 10\text{nm}$ and $500\pm 10\text{nm}$ collected at 535nm . DAF-2 DA was loaded using a single bolus $150\mu\text{l}$ ($1\mu\text{mol}$) injection. Changes in NO-dependent fluorescence were determined using the NO donor sodium nitroprusside (SNP: $100\mu\text{M}$), NO-dependent vasodilator bradykinin (BK: $100\mu\text{M}$) and non-specific NO synthase inhibitor N^{G} -nitro-L-arginine (LNA: $200\mu\text{M}$) before and after loading hearts with DAF-2 DA. Before loading, these agents did not alter epicardial fluorescence. After loading, SNP, BK and LNA produced a consistent change in each excitation wavelength. Together this suggests that change in fluorescence represents changes in the level of NO. SNP and BK increased whilst LNA significantly decreased left ventricular epicardial NO-dependent fluorescence. At the excitation wavelength of 490nm , SNP and BK increased fluorescence by $104.7\pm 18.7\text{mV}$ ($1.1\pm 0.2\%$) and $150.7\pm 26.1\text{mV}$ ($1.5\pm 0.3\%$) respectively, whilst LNA significantly decreased fluorescence by $90.3\pm 17.0\text{mV}$ ($-0.9\pm 0.2\%$). Changing the rate of aortic perfusion did not alter fluorescence suggesting that changes in aortic perfusion pressure *per se* do not contribute to the changes in DAF-2 fluorescence seen with SNP, BK or LNA. Our data suggests that DAF-2 DA is a useful fluorescence indicator for measuring NO activity in isolated hearts.

Introduction

Since the discovery that nitric oxide (NO) is enzymatically synthesised in mammalian cells, its important role has been shown in a multitude of biological systems. Apart from the well-established role of NO in vascular tone (Furchgott & Zawadzki, 1980), NO has been implicated to play a physiological role in heart rate (Herring *et al.*, 2002) and contractile regulation (Cotton *et al.*, 2001) as well having a pathological role in myocardial disease (Massion *et al.*, 2003) and a protective role against ischaemia-reperfusion injury (Jones & Bolli, 2006).

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NO is synthesised from L-arginine by three distinct isoforms of nitric oxide synthase (NOS): neuronal (nNOS) or NOS 1, inducible (iNOS) or NOS 2 and endothelial (eNOS) or NOS 3, that can be found throughout the heart (Faber-Zuschratter & Wolf, 1994;Hecker *et al.*, 1994;Rothe *et al.*, 1998;Xu *et al.*, 1999). NO may therefore have several distinct roles in cardiac muscle that is highly dependent on cellular localisation. Despite many studies, there are conflicting data on the role of cardiac NO (Sears *et al.*, 2004). For further understanding of the role of NO in myocardial function, the direct measurement of NO is desirable.

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NO is a free radical with an ultra-short half-life, highly diffusible and reactive upon formation. These properties limit understanding of its role in physiological processes as NO is difficult to measure. Attempts to measure NO levels in the whole heart have been made using electron paramagnetic resonance (Zweier *et al.*, 1995), direct assessment of total NO release in coronary effluent using chemiluminescence (Tsukada *et al.*, 2003) and with a NO sensitive probe (Kupatt *et al.*, 1997) but these techniques cannot be used in conjunction with studies on electrophysiological and mechanical events during the cardiac cycle and only total NO activity is reflected. Direct beat-to-beat quantification in the whole heart has been performed using a porphyrinic microsensor (Pinsky *et al.*, 1997) but this method is technically challenging and could cause local damage limiting interpretation of data. A more practical method that allows NO to be measured in the beating heart together with simultaneous electrophysiological and mechanical events is lacking.

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Recent developments of fluorescence indicators specific for NO offers the possibility of directly “visualising” NO in living cells with high sensitivity allowing semiquantitative analysis of NO activity (Kojima *et al.*, 1998;Sutherland *et al.*, 2001;Rodriguez *et al.*, 2005). One such NO sensitive indicator is 4,5-diaminofluorescein (DAF-2) (Kojima *et al.*, 1998). The membrane-permeable diacetate form (DAF-2 DA) has been used for the detection and imaging of NO from a range of non-cardiac cells and tissues (Broillet *et al.*, 2001;Kashiwagi *et al.*, 2002;Swindle *et al.*, 2004;Kasim *et al.*, 2001;Pittner *et al.*, 2003;Rodriguez *et al.*, 2005). Using flow cytometry, DAF-2 DA has been used in isolated cardiomyocytes (Strijdom *et al.*, 2004) but this method is not possible

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in the whole heart. To our knowledge DAF-2 DA has not been used in the whole heart. The aim of this study was to develop the technique to establish the use of DAF-2 DA in the isolated Langendorff perfused rabbit heart. To this end, the study was designed with manipulation of NO activity in the beating heart using standard NO-dependent agents and physiological interventions. Some of this data has been published in abstract form (Patel *et al.*, 2005).

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Methods and materials

Isolated Langendorff perfused beating heart preparation

Adult male New Zealand White rabbits (2.0-2.5 kg, n=8) were sacrificed with an overdose of pentobarbitone sodium (Sagatal, Rhone Merieux, Harlow, UK; 60mg, i.v.) containing Heparin (500IU, Multiparin, Wrexham, UK) via the marginal ear vein. The hearts were rapidly excised and the ascending aorta connected to the perfusion apparatus and retrogradely perfused in constant flow (35ml/min) Langendorff mode using a Gilson Minipulse 3 peristaltic pump (Anachem, Luton, UK). The heart was perfused with Tyrode solution of the following composition (mM): Na^+ : 138.0, K^+ : 4.0, Ca^{2+} : 1.8, Mg^{2+} : 1.0, HCO_3^- : 24.0, H_2PO_4^- : 0.4, Cl^- : 121.0, glucose: 11.0 and acetate: 20.0. The solution was continuously bubbled with 95 % O_2 – 5 % CO_2 to maintain a constant pH of 7.4. Temperature was maintained at 37 °C. A 3F polypropylene catheter (Portex, Kent, UK) was inserted at the apex of the left ventricle for drainage of thebesian venous effluent. Intraventricular left ventricular pressure (LVP) was monitored with a fluid filled latex balloon connected to a pressure transducer (MTL0380, ADInstruments Ltd, Chalgrove, UK) and inserted into the left ventricle via the left atrium. Left ventricular end diastolic pressure was adjusted to zero. Aortic perfusion pressure (AP) was monitored with a second pressure transducer connected in series with the aortic cannula. All procedures were undertaken in accordance with ethical guidelines set out by the UK Animals (Scientific Procedures) Act 1986 and conformed to the Guide for the Care and Use of Laboratory Animals Published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

Fluorescence measurement system

An Optoscan spectrophotometer modular system (Cairn Research Ltd, Faversham, UK) was used to measure epicardial fluorescence.. A bifurcated light guide with 2 concentrically arranged sets of fibres was used to provide excitation light to (via the central fibres) and collect emitted fluorescence from the heart (via the peripheral fibres). The light guide was placed on the left ventricular free wall and epicardial fluorescence was measured using excitation wavelengths alternating at $470\pm 10\text{nm}$ (F_{470}), $480\pm 10\text{nm}$ (F_{480}), $490\pm 10\text{nm}$ (F_{490}), and $500\pm 10\text{nm}$ (F_{500}) for 20ms, 20ms, 8ms and 2ms respectively. With the gain settings the same, these durations were chosen to correct for the fluorescence levels at the different wavelengths so that the signals could be recorded in a comparable scale. The PMT voltage settings were adjusted prior to each experimental protocol to optimal fluorescence levels to examine the effects of each manoeuvre. Emission wavelengths were collected at 535nm with a 50nm bandpass filter. A variety of excitation wavelengths were studied to determine the optimal wavelength for NO detection. The light guide was positioned perpendicular to

the epicardial surface midway between the apex and base of the heart and removed and repositioned between protocols to prevent damage to the left ventricular wall from excessive pressure.

DAF-2 loading

Hearts were perfused with Tyrode containing 0.3mM probenecid (Sigma-Aldrich, Dorset, UK) prior to loading with DAF-2 as this agent has been shown to prevent intracellular leakage of fluorescence indicators (Di *et al.*, 1990). After a period of stabilisation (30 min), DAF-2 DA (150µl, 1µmol, Calbiochem, Nottingham, UK) was injected into the side arm of the aortic cannula. An equilibration period of 60 minutes was allowed for intracellular esterases to cleave DAF-2 DA into its active form before baseline fluorescence was monitored and the experiment began.

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Experimental protocols

1) Effects of manipulation of NO activity on epicardial fluorescence

Hearts were perfused with agents that increase and decrease NO activity and the effects on epicardial fluorescence were compared before DAF-2 loading and after DAF-2 loading. The NO donor sodium nitroprusside (SNP: 100µM, Sigma-Aldrich, Dorset, UK) and endothelial NO-dependent vasodilator bradykinin (BK: 100µM, Sigma-Aldrich) were used as positive modulators of NO activity whilst the non-specific NO synthase inhibitor N^G-nitro-L-arginine (LNA: 200µM, Cyclopps Biochem, Salt Lake City, Utah, US) was used to reduce endogenous NO production. Fluorescence was continuously measured before, during and after perfusion (washout) with these agents. SNP was also tested in the presence of the mechanical uncoupler diacetyl monoxime (BDM, 20mM, Sigma-Aldrich) to assess the contribution of movement artefact and ventricular contraction to the fluorescence signals.

2) Effects of change in perfusion on epicardial fluorescence

As the agents used to alter NO activity have vasoactive properties that affect aortic perfusion, experiments were carried out to examine the effect of perfusion pressure change on NO-dependent fluorescence. The changes in aortic perfusion pressure resulting from SNP, BK and LNA were mimicked by altering the rate of perfusion and the effect on DAF-2 fluorescence examined. The rate of perfusion was increased from 35.4±0.2ml/min to 38.6±0.2ml/min to match the change in perfusion pressure with LNA and separately decreased to 32.2±0.2ml/min to match the change in perfusion pressure with SNP and bradykinin for a period of 5 minutes.

Signal measurement and analysis

All signals were recorded using a Powerlab 800/s system and digitised at 1kHz using Chart software (ADInstruments Ltd). Data were stored and displayed on a Power Macintosh G3 personal computer

(Apple, UK). Left ventricular pressure (LVP) and aortic perfusion pressure (AP) was continuously monitored and analysed simultaneously with all fluorescence signals

There was a cyclical variation of the emitted fluorescence collected at each excitation wavelength. The variation in the signal was similar before and after loading with DAF-2. Fig 1A shows the beat-by-beat cyclical changes in epicardial fluorescence in a typical experiment after DAF-2 loading. Detailed analysis of preliminary data (Patel *et al*, 2005) showed that both the minimum and maximum level of the fluorescence signal, correlating to systole and diastole respectively, at each wavelength varied to similar degrees with manoeuvres that increased or decreased fluorescence. For clarity, the mean level will be presented in this study for all fluorescence data. With the experimental settings detailed above, fluorescence was highest at the excitation wavelength of F₄₉₀ followed by F₄₈₀, F₅₀₀ then F₄₇₀. The baseline cyclical variation in fluorescence was abolished in the presence of the mechanical uncoupler BDM as highlighted in the aligned profiles of Fig 1 Bi and ii. These data highlight the contribution of movement artefact that is likely caused by factors accompanying contraction of the ventricle against the light guide, to the beat-to-beat cyclical variation in the fluorescence signal.

Statistical analysis

All data are expressed as mean $\pm$ SEM and analysed using repeated measures ANOVA with Tukey's post hoc analysis where appropriate. P<0.05 was considered significant.

Results

I) Pharmacological manipulation of NO

Prior to loading of DAF-2 DA

After stabilisation of the isolated hearts, the effects of pharmacological manipulation of the level of NO on epicardial fluorescence was tested prior to DAF-2 loading in 6 of the 8 hearts used. Fluorescence measured at all excitation wavelengths, did not change after perfusion with the NO donor sodium nitroprusside (SNP), endothelial NO-dependent vasodilator bradykinin (BK) and non-specific NO synthase inhibitor LNA (Table 1) in the absence of DAF-2 DA loading. However, SNP and BK significantly decreased whilst LNA significantly increased aortic perfusion pressure (Table 1). LVP was significantly decreased during SNP perfusion and LNA perfusion, whilst heart rate was not significantly altered with any agent (Table 1).

Loading the hearts with DAF-2 DA had a significant effect on left ventricular (LVP) and aortic perfusion (AP) pressures. LVP decreased from 74.8 ± 4.1 mmHg to 52.5 ± 4.3 mmHg ($P < 0.05$ vs. pre loading) whilst AP was increased from 61.3 ± 4.8 mmHg to 75.9 ± 6.0 mmHg ($P < 0.001$ vs. pre-loading).

After loading of DAF-2 DA

Effect of SNP

The effect of SNP on left ventricular pressure, aortic perfusion pressure and fluorescence at each wavelength in a typical experiment after DAF-2 loading is illustrated in Figure 2A. Mean data from all experiments ($n=8$) showed that SNP caused a significant and reversible decrease in aortic perfusion pressure from 73.4 ± 7.9 mmHg to 67.1 ± 7.7 mmHg ($P < 0.05$) and LVP (53.5 ± 3.8 mmHg to 46.2 ± 4.4 mmHg, $P < 0.05$) whilst heart rate was not significantly changed (173.5 ± 9.1 bpm to 177.1 ± 8.3 bpm, $P > 0.05$).

DAF-2 fluorescence increased during SNP perfusion and returned to pre-SNP levels after washout to Tyrode solution. Perfusion with SNP produced a small but highly significant and reversible increase in fluorescence at each excitation wavelength. Fluorescence was increased from a baseline of 3.815 ± 0.088 V (F_{470}), 8.957 ± 0.206 V (F_{480}), 9.490 ± 0.233 V (F_{490}) and 7.623 ± 0.058 V (F_{500}) to 3.840 ± 0.090 V (F_{470} , $P < 0.05$ vs baseline), 9.037 ± 0.212 V (F_{480} , $P < 0.01$ vs baseline), 9.594 ± 0.240 V (F_{490} , $P < 0.01$ vs baseline) and 7.640 ± 0.051 V (F_{500} , $P < 0.05$ vs baseline) and returned to baseline on washout (data not shown). The increase in fluorescence was significantly larger at F_{480} and F_{490} when compared to F_{470} and F_{500} (Fig 2 B+C) without affecting the amplitude of the fluorescence signal.

Effect of SNP in the presence of the mechanical uncoupler BDM

As mentioned above, the cyclical variation in epicardial fluorescence was postulated to be due to movement artefact. It is therefore possible that movement artefact may also contribute to the changes seen from pharmacological manipulation of NO activity. To assess this, the effect of perfusion of Tyrode containing SNP was re-tested in the presence of the mechanical uncoupler diacetyl monoxime (BDM) in 6 hearts (Fig 3A). BDM significantly inhibited contractile activity and caused a decrease in aortic perfusion pressure (74.0 ± 6.2 mmHg to 66.4 ± 4.7 mmHg). Perfusion with SNP did not restore mechanical activity but caused a small non-significant increase in aortic perfusion pressure compared to BDM alone (70.0 ± 4.4 mmHg, $P > 0.05$).

Fluorescence at each wavelength studied was reduced significantly ($P < 0.01$) during BDM perfusion (Fig 4A) from a baseline of 3.874 ± 0.027 V to 3.828 ± 0.034 V (F_{470}), 9.116 ± 0.040 V to 9.005 ± 0.048 V (F_{480}), 9.734 ± 0.085 V to 9.592 ± 0.079 V (F_{490}) and 7.690 ± 0.015 V to 7.669 ± 0.016 V (F_{500}). At the same time, the cyclical variation in fluorescence was greatly reduced at all wavelengths from 46.8 ± 8.3 mV to 21.8 ± 3.0 mV (F_{470} , $-50.4 \pm 6.3\%$), 101.2 ± 20.0 mV to 37.0 ± 3.9 mV (F_{480} , $-58.5 \pm 7.2\%$), 110.7 ± 21.5 mV to 37.2 ± 5.3 mV (F_{490} , $-64.2 \pm 3.8\%$) and from 21.0 ± 3.7 mV to 13.5 ± 1.5 mV (F_{500} , $-30.8 \pm 8.6\%$). In the presence of BDM, SNP perfusion significantly ($P < 0.001$) increased mean DAF-2 fluorescence to 3.884 ± 0.029 (F_{470}), 9.148 ± 0.031 (F_{480}), 9.758 ± 0.074 (F_{490}) and 7.692 ± 0.013 (F_{500}). There was a trend for all fluorescence values to return towards pre-SNP levels on returning back to Tyrode solution containing BDM alone. The change in fluorescence during perfusion with BDM and SNP was significantly larger at F_{480} and F_{490} when compared to F_{470} and F_{500} (Fig 3 B+C).

Effect of BK

An example of the effect of the endothelial NO-dependent vasodilator bradykinin (BK) is illustrated in Fig 4A. BK produced a reversible decrease in aortic perfusion pressure from 72.6 ± 7.4 mmHg to 68.2 ± 7.1 mmHg ($P < 0.05$, $n=6$). LVP (52.6 ± 4.6 mmHg to 50.0 ± 4.8 mmHg) and heart rate (166.3 ± 8.6 bpm to 166.9 ± 8.4 bpm) were unaffected. During BK, fluorescence at each wavelength was increased from a baseline of 3.910 ± 0.082 V (F_{470}), 9.228 ± 0.098 V (F_{480}), 9.773 ± 0.073 V (F_{490}) and 7.674 ± 0.019 V (F_{500}) to 3.963 ± 0.085 V (F_{470} , $P < 0.01$ vs baseline), 9.359 ± 0.108 V (F_{480} , $P < 0.01$ vs baseline), 9.924 ± 0.065 V (F_{490} , $P < 0.01$ vs baseline) and 7.694 ± 0.017 V (F_{500} , $P < 0.01$ vs baseline) and returned to baseline on washout (data not shown). The increase in fluorescence was significantly larger at F_{480} and F_{490} when compared to F_{470} and F_{500} (Fig 4 B+C) without altering the amplitude of each wavelength.

Effect of LNA

Figure 5 illustrates the effect of perfusion with the non-specific NO synthase inhibitor N^G-nito-L-arginine (LNA) on aortic perfusion pressure, left ventricular pressure and fluorescence at each wavelength studied. Data from 8 hearts demonstrated that LNA significantly increased aortic perfusion pressure (72.8±8.7mmHg to 76.3±9.2mmHg, P<0.05) whilst significantly decreasing left ventricular pressure (50.1±5.4mmHg to 45.4±5.2mmHg, P<0.05). Heart rate remained unchanged (179.5±4.9bpm to 182.6±7.0bpm, P>0.05). LNA significantly reduced fluorescence from a baseline of 4.041±0.101 V to 4.000±0.1000 V (F₄₇₀, P<0.01 vs baseline), 9.374±0.170 V to 9.290±0.167 V (F₄₈₀, P<0.01), 9.865±0.126 V to 9.775±0.124 V (F₄₉₀, P<0.01) and 7.709±0.018 V to 7.697±0.017 V (F₅₀₀, P<0.01) and returned to baseline on washout (data not shown). Once again there was a significant wavelength dependent difference in the magnitude of change with the largest decreases in fluorescence seen at the excitation wavelengths of F₄₈₀ and F₄₉₀ when compared to F₄₇₀ and F₅₀₀ (Fig 5 B+C).

II) Altering the rate of perfusion

The effect of directly changing the rate of perfusion, thereby altering aortic perfusion pressure, on the various parameters in the presence of DAF-2 is illustrated in Figure 6 and the mean data are shown in Table 2 (n=6). Increasing the rate of perfusion (from 35.4±0.2ml/min to 38.6±0.2ml/min) significantly increased aortic perfusion pressure whilst decreasing the rate of perfusion (to 32.2±0.2ml/min) had the opposite effect. Increasing and decreasing the rate of perfusion significantly increased and decreased LVP respectively whilst heart rate was significantly decreased by decreasing the rate of perfusion but not affected by increasing the rate of perfusion. As indicated in Table 2, neither increasing nor decreasing the rate of perfusion, altered left ventricular epicardial fluorescence either before or after loading with DAF-2 DA.

Discussion

To the best of our knowledge, the current study is the first to use DAF-2 DA to reflect changes in NO fluorescence in the isolated beating heart. This technique has many advantages over other technically challenging methods that measure NO. Firstly, the heart is loaded with a single bolus injection of the indicator that is quick and far less cumbersome than other loading techniques. Secondly, the NO-dependent signal can be recorded from multiple sites over the surface of the beating heart without detrimental effects to myocardial contraction. Thirdly and perhaps the strongest advantage is that the NO signal can be associated with electrical and mechanical events.

Measuring NO-dependent fluorescence in the whole heart

Our results support the notion that the changes seen in epicardial fluorescence with pharmacological manipulation of NO activity were due to changes in [NO] in the beating heart. Firstly, although the changes induced by SNP, BK and LNA in the fluorescence signal at 470–500nm are small relative to the large background NO signal, they were highly significant, reproducible and reversible. This is supported by the knowledge that DAF-2 selectively traps NO between two of its amino acid groups yielding the highly fluorescent compound triazolofluorescein (DAF-2T) that fluoresces when excited between 480-500nm (Kojima *et al.*, 1998). Studies on the *in vitro* excitation spectrum of DAF-2 have shown a peak fluorescence at 500nm with little shift in the spectra at different NO concentrations (Kojima *et al.*, 1998). In our study, 4 different excitation wavelengths were used (470nm, 480nm, 490nm and 500nm) to determine the optimal wavelength to investigate NO activity in the whole heart. Changes were seen at all excitation wavelengths studied in association with manipulation of NO but the fluorescence changes with 480nm and 490nm were much larger than with 470nm or 500nm. The difference from *in vitro* data may be due to a number of factors especially considering the different biological conditions and optical path in the whole heart as compared to *in vitro* cuvette experiments (Bassani *et al.*, 1995). Hence, excitation wavelengths of 480 nm and 490 nm are most suitable for measuring NO activity in the isolated heart using DAF-2. Secondly, the changes in fluorescence were accompanied with directionally opposite changes in aortic perfusion pressure, which grossly represent the level of coronary vascular tone. These changes in perfusion pressure are in line with the known vascular effects of NO, i.e. promotion of NO activity and vasodilatation from NO donors / bradykinin and reduction of NO production and vasoconstriction from endothelial NO synthase inhibitors.

Thirdly, there was no change in fluorescence in the absence of DAF-2 suggesting that changes observed were not the result of an alteration in the level of background 'auto' fluorescence. In addition, physical manipulation of the rate of perfusion with a resultant change in perfusion pressure

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similar to that caused by the pharmacological agents used, did not alter DAF-2 fluorescence. Therefore the change in fluorescence observed in the presence of DAF-2 is unlikely to have arisen from the vasoactive effects of SNP, BK and LNA *per se* but more likely to be the result of changes in the level of NO that precede the increase or decrease in aortic perfusion pressure.

The direction of the change in the fluorescence signal was consistent with the pharmacological actions of SNP, BK and LNA involving the nitric oxide – cyclic guanosine monophosphate (cGMP) pathway. SNP donates NO that would trigger an increase in cGMP within smooth muscle cells and in turn reduce intracellular Ca^{2+} influx reducing vascular tone, resulting in vasodilatation. BK activates endothelial BK type 2 receptors stimulating the production of NO (Wiemer *et al.*, 1991). In turn, NO diffuses to vascular smooth muscle cells increasing cGMP production, inhibiting calcium influx in smooth muscle cells resulting in the lowering of vascular tone. BK also activates ventricular intrinsic neurones (Waldmann *et al.*, 2006) and subject to the presence of NO synthase, may contribute to the increase in DAF-2 fluorescence with BK. The effect of LNA would reduce the production of NO and produce opposite effects to SNP and BK, and this was clearly reflected by the direction of changes in fluorescence.

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DAF-2 fluorescence is Ca^{2+} concentration dependent (Broillet *et al.*, 2001b). In this latter study, increasing Ca^{2+} *in vitro* or stimulating intracellular calcium release in embryonic kidney cells did not alter ongoing DAF-2 fluorescence. This increase in Ca^{2+} concentration did however change DAF-2 fluorescence during NO donation, suggesting that Ca^{2+} concentration increases the sensitivity of DAF-2 for NO. This is in contrast to other studies which suggest that DAF-2 fluorescence is insensitive to physiological Ca^{2+} concentration (Gomes *et al.*, 2006). This aspect was not directly examined in our study. However, left ventricular pressure, an indicator of Ca^{2+} handling, was measured during manipulation of NO. There were minor changes in LVP with SNP and LNA occurring in parallel with changes in DAF-2 fluorescence yet no significant change in LVP during BK despite significant changes in fluorescence. Therefore it is unlikely that Ca^{2+} concentration contributes significantly to the fluorescence changes seen during pharmacological manipulation of NO in our data.

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Origins of basal NO signal

LNA decreased DAF-2 fluorescence suggesting that there is basal NO activity present in the isolated heart and is supported by others (Paz *et al.*, 2003; Pabla & Curtis, 1996; Tsukada *et al.*, 2003; Kupatt *et al.*, 1997). It is likely that the basal fluorescence signal could originate from a number of sources since NO synthases can be found in endothelial cells (Miethke *et al.*, 2003), neurones and nerve fibres of cardiac ganglia (Sosunov *et al.*, 1996; Sosunov *et al.*, 1995) and subcellularly located within cardiac myocytes e.g. sarcoplasmic reticulum and mitochondria (Xu *et*

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al., 1999; Kanai *et al.*, 2001). We have performed preliminary experiments applying this novel NO measurement technique to the innervated isolated heart preparation which we developed (Ng *et al.*, 2001). In these experiments (Patel *et al.*, 2006), the non-specific NO synthase inhibitor LNA reduced basal NO signal whereas the specific inhibitor of neuronal NO synthase, TRIM did not. Direct vagus nerve stimulation was shown to release NO in the ventricle which was measured by DAF-2 thus highlighting the physiological application of the fluorescence technique. Both LNA and TRIM inhibited NO production from vagus nerve stimulation. Further studies are required to elucidate the precise tissue compartments from which NO is produced by the different specific NO synthases.

Although NO release from shear stress can potentially contribute to basal NO production, this is unlikely to have contributed significantly to the NO signal measured in the current study since physically manipulating the rate of perfusion did not alter epicardial DAF-2 fluorescence despite significant changes in perfusion pressure. The signal resulting from NO production stimulated by shear stress may have been too weak to be detected by this method.

Motion artefact

A major problem in attempting to measure changes in a beating heart is the degree of movement due to contraction. Examining the effect of SNP in the presence of the mechanical uncoupler BDM allowed assessment of the contribution of myocardial contraction. BDM reduced the level and amplitude of all fluorescence signals recorded Fig 1B (i and ii) and Fig 3, supporting the notion that the main contributing factor to the cyclical variation in the fluorescence signal is motion artefact. Regardless of the contribution from mechanical activity on DAF-2 fluorescence, ventricular contraction did not affect the SNP evoked increases in fluorescence. SNP produced a comparable increase in DAF-2 fluorescence with and without BDM suggesting that the changes in fluorescence with SNP reflect changes in the level of NO and that the pharmacological induced changes in fluorescence are separate from the motion artefacts.

Limitations

The lack of calibration of [NO] represents a limitation using DAF-2 fluorescence in isolated hearts. This is technically challenging and requires the quenching of fluorescence to obtain minimum fluorescence and increasing the fluorescence to a known maximum concentration of NO. Even in more established fluorescence measurement methods such as Ca^{2+} transients, calibrating the intracellular concentration from fluorescence measurements in the whole heart poses significant difficulties and is subject to a multitude of confounding factors and errors (Bassani *et al.*, 1995). However, the ability to measure fluorescence in the whole heart outweighs this limitation enabling changes in NO to be directly studied with electrical and mechanical events. Studying NO activity in

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the whole heart precludes specific intracellular compartments being interrogated but the activity of different NO synthase isoforms and the contribution to the NO fluorescence signal can be studied using isoform specific modulators as mentioned above.

Conclusions

We have shown in this study a new method of measuring NO activity in the isolated beating heart using the NO fluorescence indicator DAF-2 DA. Loading of the indicator using a single bolus injection was straightforward and resulted in NO-dependent fluorescence which was verified by reproducible changes in NO activity using pharmacological agents. This method may prove invaluable in investigating the role of NO in physiology and pathophysiology in the whole heart.

Acknowledgment

A British Heart Foundation Project Grant (PG/02/088) supported this study.

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Figure 1

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Figure 1. A) Example of the change in left ventricular pressure (LVP), aortic perfusion pressure, and epicardial fluorescence at excitation wavelengths of 470nm (F_{470}), 480nm (F_{480}), 490nm (F_{490}) and 500nm (F_{500}) in a typical experiment after loading of DAF-2 DA. Heart was paced via the right ventricle at 300ms cycle length. B) LVP, aortic perfusion pressure and F_{490} over one cardiac cycle from A in (i) and a separate experiment in the presence of the mechanical uncoupler diacetyl monoxime (20mM) (ii). The vertical dashed lines represent diastole and systole respectively.

Figure 2. (A) Left ventricular pressure (LVP), aortic perfusion pressure (AP) and fluorescence at excitation wavelengths of 470nm (F_{470}), 480nm (F_{480}), 490nm (F_{490}) and 500nm (F_{500}) during perfusion of Tyrode containing 100 μ M of the NO donor sodium nitroprusside (SNP). Data representing the change in fluorescence in millivolts (B) and per cent change (C). Mean $\pm$ SEM, n=8. **P<0.01, *** P<0.001 vs. BL, ANOVA.

Figure 3. (A) Left ventricular pressure (LVP), aortic perfusion pressure (AP) and fluorescence at excitation wavelengths of 470nm (F_{470}), 480nm (F_{480}), 490nm (F_{490}) and 500nm (F_{500}) during perfusion of Tyrode containing 20mM diacetyl monoxime (BDM) followed by perfusion with the NO donor sodium nitroprusside (SNP, 100 μ M). Data representing the change in fluorescence in millivolts (B) and per cent change (C). Mean $\pm$ SEM, n=6, *P<0.05, *P<0.01, **P<0.01 and *** P<0.001 comparisons indicated by the horizontal bars, ANOVA.

Figure 4. (A) Left ventricular pressure (LVP), aortic perfusion pressure (AP) and fluorescence at excitation wavelengths of 470nm (F_{470}), 480nm (F_{480}), 490nm (F_{490}) and 500nm (F_{500}) during perfusion of Tyrode containing 100 μ M of the NO-dependent vasodilator bradykinin (BK). Data representing the change in fluorescence in millivolts (B) and per cent change (C). Mean $\pm$ SEM, n=6, *P<0.05, **P<0.01, *** P<0.001 vs. BL, ANOVA.

Figure 5. (A) Left ventricular pressure (LVP), aortic perfusion pressure (AP) and fluorescence at excitation wavelengths of 470nm (F_{470}), 480nm (F_{480}), 490nm (F_{490}) and 500nm (F_{500}) during perfusion of Tyrode containing 200 μ M of the non-specific NO inhibitor N^G-nitro-L-arginine (LNA). Data representing the change in fluorescence in millivolts (B) and per cent change (C). Mean $\pm$ SEM, n=8, *P<0.05, **P<0.01, *** P<0.001 vs. BL, ANOVA.

Figure 6. Left ventricular pressure (LVP), aortic perfusion pressure (AP) and fluorescence at excitation wavelengths of 470nm (F_{470}), 480nm (F_{480}), 490nm (F_{490}) and 500nm (F_{500}) in response to an increase (A) and decrease (B) in the rate of perfusion.