

NASA Grant NAGW-4256 Final Progress Report

Pulsatile Fluid Shear in Bone Remodeling

Abstract:

The objective of this investigation was to elucidate the sensitivity to transients in fluid shear stress in bone remodeling. Bone remodeling is clearly a function of the local mechanical environment which includes interstitial fluid flow. Traditionally, load-induced remodeling has been associated with low frequency (1-2 Hz) signals attributed to normal locomotion. McLeod and Rubin, however, demonstrated *in vivo* remodeling events associated with high frequency (15-30 Hz) loading. Likewise, other *in vivo* studies demonstrated that slowly applied strains did not trigger remodeling events. We therefore hypothesized that the mechanosensitive pathways which control bone maintenance and remodeling are differentially sensitive to varying rates of applied fluid shear stress.

Using nitric oxide (NO), prostaglandin E₂ (PGE₂) and c-fos as markers, we have investigated the role of transients in fluid shear stress in bone remodeling. We report:

1. Flow-induced NO production is biphasic. Transients associated with the start of flow activate distinct mechanosensitive pathways relative to steady and sustained flow.
2. Impulse flow stimulates c-fos activation 2 fold relative to ramped flow to the same magnitude.
3. Mechanosensitive pathways activated by step changes in flow are calcium and G-protein dependent.
4. Mechanosensitive pathways associated with sustained flow are calcium and G-protein independent.

Introduction:

Bone is a porous and compressible material, which is subjected to a rapid and substantial interstitial fluid (ISF) flow. This flow is driven by a gradient between the venous pressure in the intramedullary canal and the lymphatics at the periosteum. Mechanical loading (in particular, axial or bending loads) induces pulsatile pressure gradients which cause transients in the ISF flow. The objectives of this investigation were to develop an *in vitro* model to demonstrate the characteristics of transients in fluid flow-induced shear stress versus steady shear stress.

Utilizing a parallel plate flow chamber and either syringe or head driven fluid flow, we subjected primary rat calvarial cells to well defined fluid shear stresses. Nitric oxide and prostaglandin E₂, both osseointegrative humoral agents, were used as markers. Northern blot analysis of c-fos expression was also performed as an indicator of mitogenic activity.

Results:

Using an *in vitro* flow model with primary rat calvarial cells and a parallel plate flow chamber, we reported that fluid flow-induced shear stress is a potent stimulus of NO release in osteoblasts, with a production rate 16 fold greater than that associated with cytokine stimulation (Figure 1).

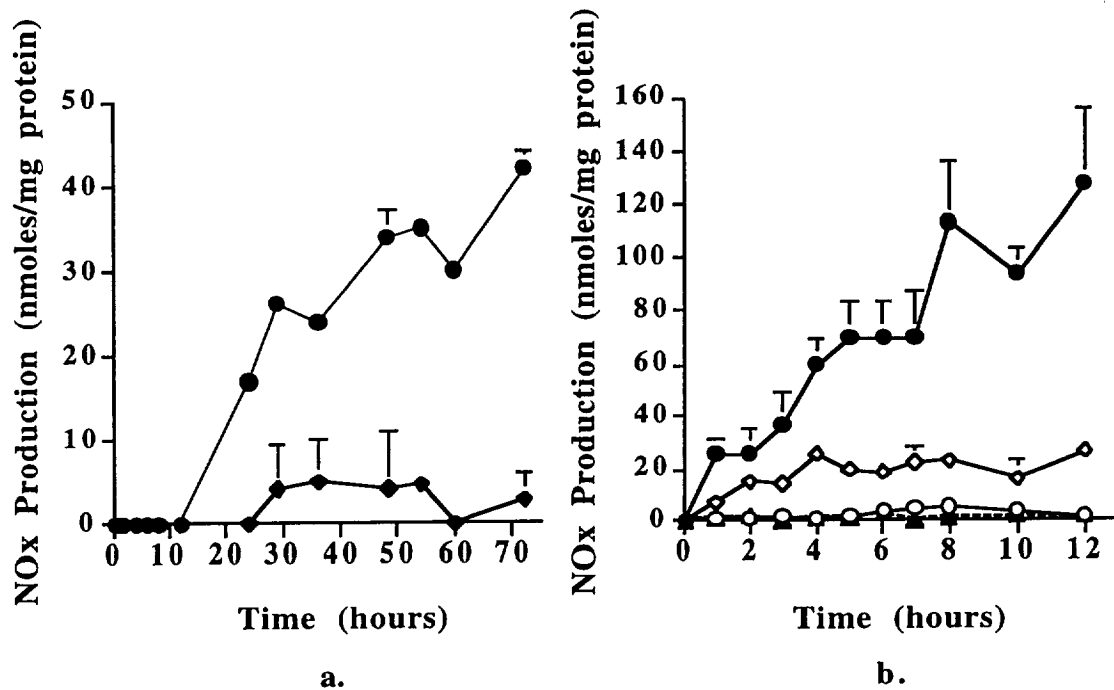


FIGURE 1a. Cytokines (\bullet) produces continuous NO release in primary rat calvarial cells (6 nmols/mg/hr) after a 12 hour lag phase of zero production. Figure 1b. Flow (\bullet) (6 dynes/cm^2) induces an immediate and sustained release (9.8 nmols/mg/hr), suggesting a constitutively present NOS isoform. $100\mu\text{M}$ L-NAA (\blacklozenge) inhibits NO production. Static controls (O) (Δ) produce negligible NO (From Johnson, McAllister and Frangos 1996).

Utilizing a chemiluminescent NO analyzer with picomolar sensitivity, we delineated the response to the start of flow (0-.5 hr) from steady flow (.5-6 hr). Flow-induced NO response demonstrates a biphasic response which we hypothesized was due to activation of distinct pathways which were sensitive to transients in shear versus steady shear. To further investigate this biphasic response, we treated cells with GDP β S, a G-protein inhibitor, and quin 2/AM, an intracellular calcium chelator. In both cases, the response to the step change in flow was significantly attenuated, while the response to sustained flow was unchanged (Figure 2).

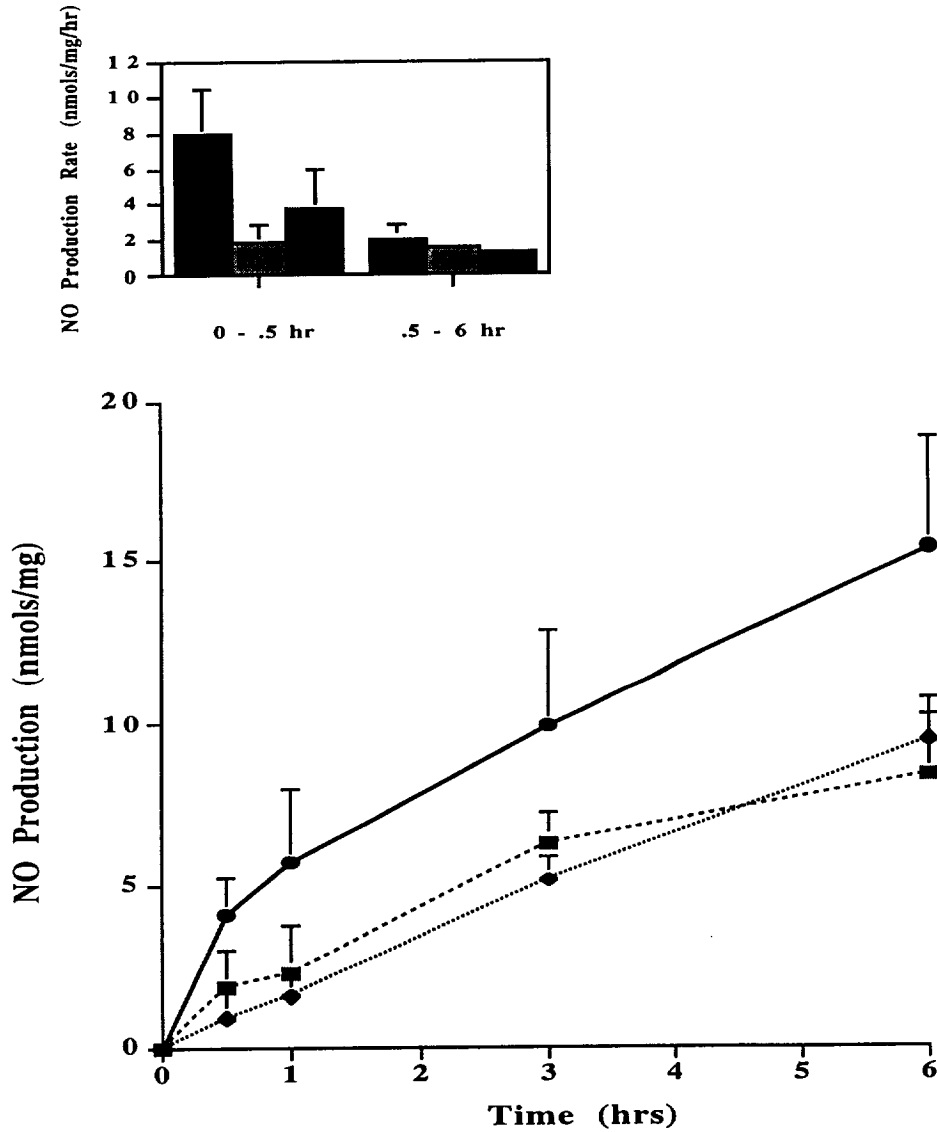


Figure 2. Fluid flow (8 dynes/cm²) (•) versus flow + quin 2/AM (30 μM) (■) and flow + GDPβS (900 μM) (◆). *Inset.* Production rates for flow (black), quin 2/AM (white) and GDPβS (gray). Flow induced NO response demonstrates a biphasic response. Chelating intracellular calcium with quin 2/AM (.5 hr preincubation) significantly inhibits initial flow response (0-.5hr) but does not affect sustained production (.5-6 hr). GDPβS (3 hr preincubation) inhibited initial flow response but did not attenuate sustained production rate.

Calcium independence in sustained production was further demonstrated by the addition of calcium ionophore A23187 to static cultures. While calcium ionophore was able to stimulate a moderate immediate response (0-.25 hr.), no sustained production was observed (Figure 3).

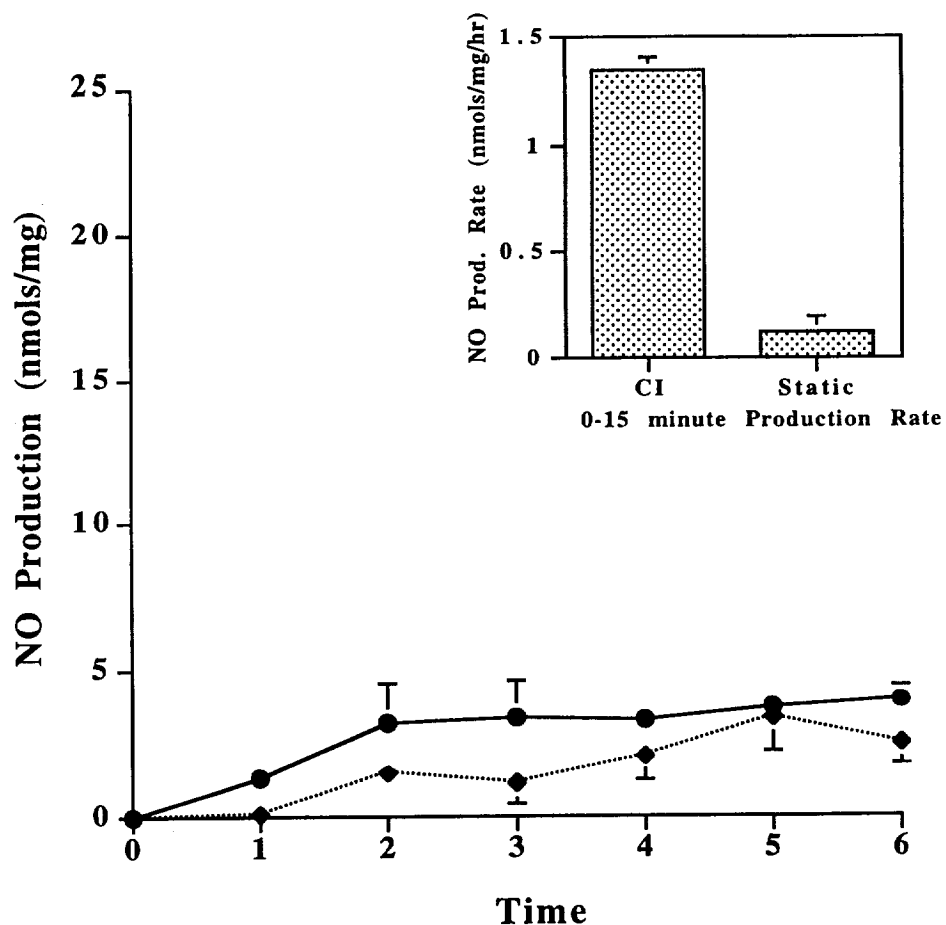


Figure 3. A23187 treatment (1µM) (●) does not stimulate sustained NO production relative to control (vehicle only) (◆) but stimulates a burst in NO production within 15 minutes (inset).

To further identify the subclass of G-proteins involved in the immediate NO response, cells were treated with pertussis toxin. PTx treated cells were not significantly different than flow alone (Figure 4). PTx did, however, significantly attenuate PGE₂ production as we had previously reported, demonstrating that the efficacy of the drug.

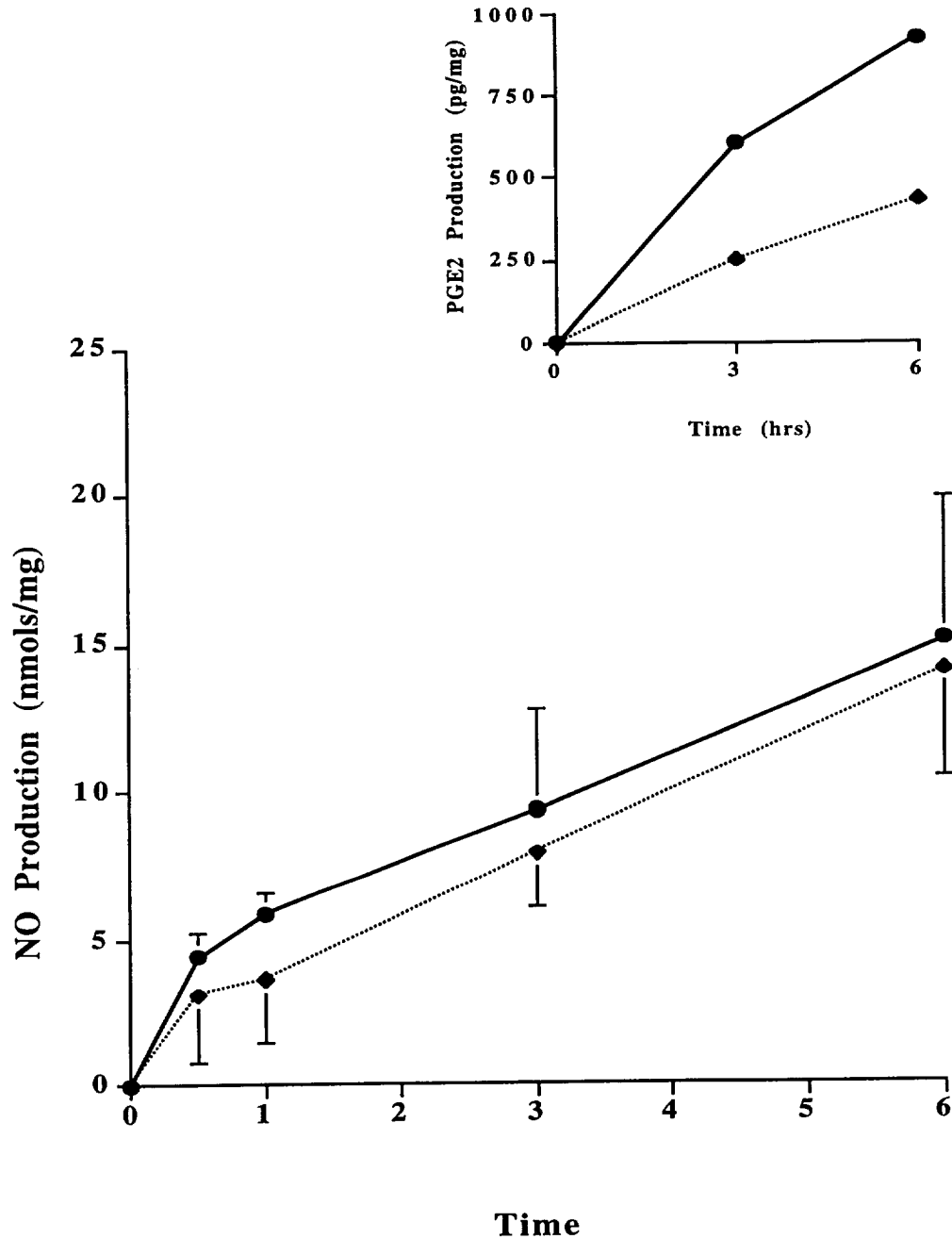


Figure 4. Pertussis toxin (1 μ g/ml) (◆) does not affect NO production rates relative to flow + vehicle (•).
Inset Ptx does inhibit flow-induced PGE₂ production.

To further demonstrate that this biphasic response was due to differences in the flow profile, we investigated *c-fos* expression as a marker of mitogenic activity. Impulse flow stimulated a 2 fold increase in *c-fos* expression relative to ramped flow to the same level of shear stress (Figure 5).

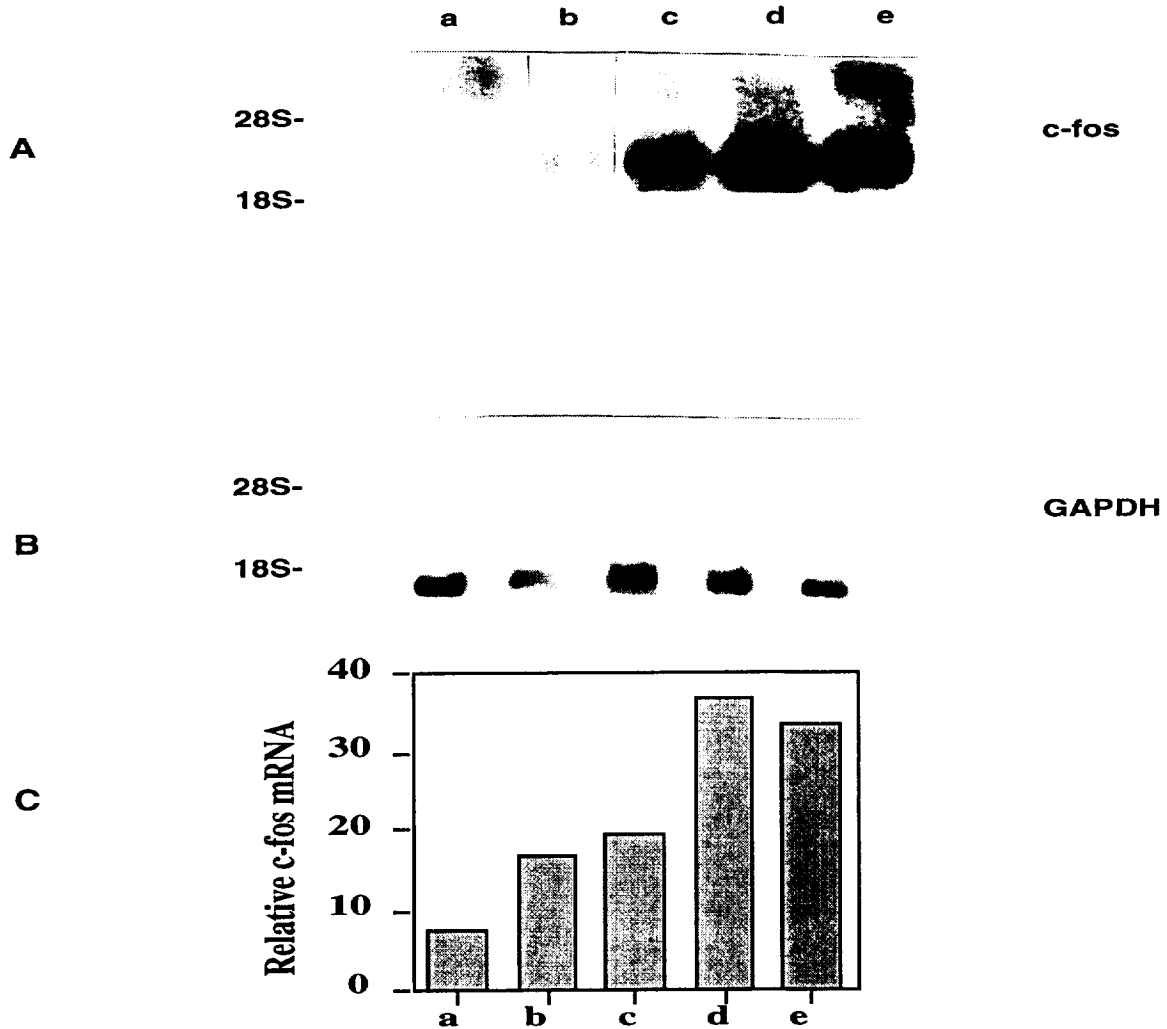


Figure 5. Ramp and step flow elicit *c-fos* expression in cultured osteoblasts (umr-106). Cells were subjected to: static control (lane a); ramp flow, where flow was smoothly increased from 0-16 dyne/cm² in 5 minutes, then held at 16 dyne/cm² for 25 minutes (lane b) or 30 minutes (lane c); step flow, where flow was instantaneously increased from 0-16 dyne/cm² and held at 16 dyne/cm² for 30 minutes (lane d); and a positive control where cells were starved for 48 hours then stimulated with 100 μM ATP for 1 hour. Isolated RNA was analyzed by Northern blot with 1.0 kb *c-fos* cDNA (panel A) and 0.8 kb GAPDH cDNA as the probe (panel B). *c-fos* expression determined by densitometry and normalized by GAPDH mRNA is shown in panel C.

Previous investigations utilizing pulsatile flow have been limited by the undefined nature of the flow profile. We have therefore developed an *in vitro* model to investigate frequency response under well defined pulsatile shear stress (Figure 6).

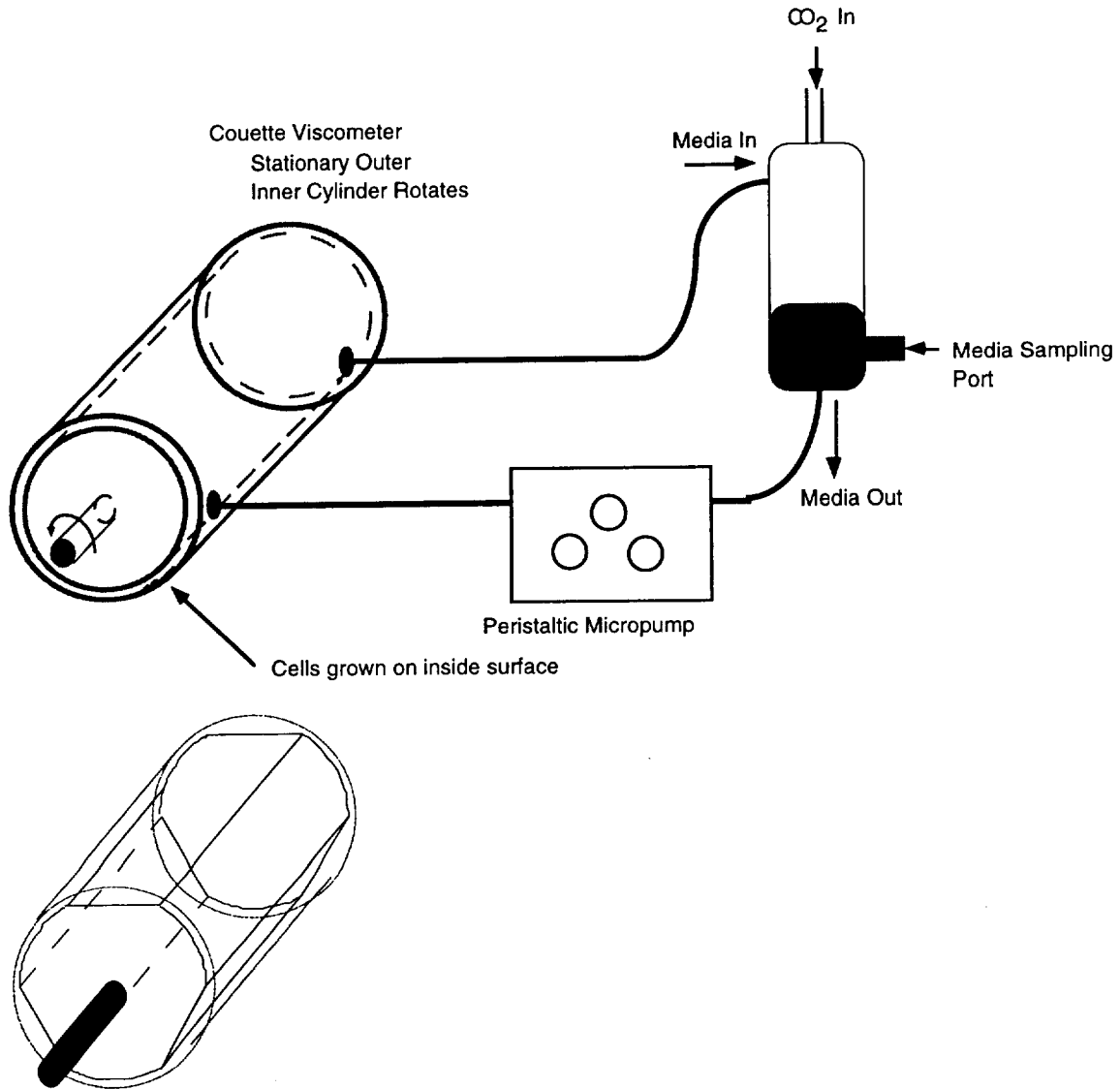


Figure 6. Schematic of couette viscometer. Cells are grown to confluence on the inner surface of the larger cylinder. Shear stress is applied by rotating the inner cylinder with a computer controlled motor while holding the outer cylinder stationary. Shear stress is proportional to fluid viscosity, gap width, and relative rotational velocity. Cells are grown in a rotating system so that they are preconditioned to a flow environment. Media is recirculated longitudinally by a micro-peristaltic pump for gas exchange and media sampling, with one volume exchange every 6 minutes. Lobed cores give a well defined pulsatile shear stress.

Conclusions:

Using nitric oxide (NO) and prostaglandin E₂ (PGE₂) and c-fos as markers, we have investigated the role of transients in fluid shear stress in bone remodeling. We report:

1. Flow-induced NO production is biphasic. Transients associated with the start of flow activate distinct mechanosensitive pathways relative to steady and sustained flow.
2. Impulse flow stimulates c-fos activation 2 fold relative to ramped flow to the same magnitude.
3. Mechanosensitive pathways activated by step changes in flow are calcium and G-protein dependent.
4. Mechanosensitive pathways associated with sustained flow are calcium and G-protein independent.

Publications:

Johnson, D.L., McAllister, T.N., and J.A. Frangos. 1996. Fluid flow stimulates rapid and continuous release of nitric oxide in osteoblasts. *Am. J. Physiol.* 271(34):E205-E208.

Reich, K.M., McAllister, T.N., Gudi, S. and J.A. Frangos. 1997. Activation of G proteins mediates flow-induced prostaglandin E₂ production in osteoblasts. *Endocrinology* 138:1014-1018.

Nulend, J.K., Van der Plas A., Semeins, C., Ajbui, N.E., Frangos, J.A., Nijweide P.J., and E.H. Burger. 1995. Sensitivity of osteocytes to biomechanical stress in vitro. *FASEB Journal.* 9:441-445.

Hillsley, M.J. and J.A. Frangos. 1996. Osteoblast hydraulic conductivity is regulated by calcitonin and parathyroid hormone. *Journal of Bone and Mineral Research.* 11:114-124.

Hillsley, M.V. and J.A. Frangos. 1994. Bone tissue engineering: the role of interstitial fluid flow. *Biotechnology and Bioengineering.* 43:573-581.

Hillsley, M.V. and J.A. Frangos. 1997. Alkaline phosphatase in osteoblasts is down regulated by pulsatile flow. *Calcif. Tissue Int.* 60:48-53.

McAllister, T.N., Moffarah, M. and J.A. Frangos. Fluid flow-induced signal transduction in osteoblasts: The role of G-proteins and calcium in steady and transient fluid flow. In preparation.