

**CHANGES IN DESMIN AND VIMENTIN CONTENTS
AFTER CLOFIBRATE TREATMENT ON RATS.**

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Summary

We analyzed *in vivo* effects of clofibrate and clofibric acid on vimentin and desmin content in rat myocardiocytes. Fifteen Sprague Dawley rats were divided in three groups of five animals each: clofibric acid, clofibrate, and untreated. After 30 days, the rats were sacrificed, the hearts excised, and the vimentin and desmin content was measured by Western blotting and densitometry. Clofibric acid treatment caused a 27.4% decrease in the amount of vimentin and a 49% decrease in desmin. With clofibrate, vimentin decreased 29.5% and desmin decreased 48.7%. Moreover, we observed alterations in the cellular organization of the filaments of desmin and vimentin in all treated animals. Intermediate filaments are known to play a role in the anchoring and cellular organization of myocardiocytes. The adverse effects of clofibrate and clofibric acid on patients may be partially due to decreased desmin and vimentin levels.

Keywords: Clofibrate, clofibric acid, myocardiocytes, rat, vimentin, desmin.

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Introduction

The prevention of cardiovascular diseases is an important aim in public health. As hyperlipidaemia is one of the main causes of cardiovascular disease, a number of clinical trials have involved the testing of hypolipidaemic drugs, i.e. statins, colestyramine, gemfibrozil and clofibrate. Clofibrate is used to lower high levels of cholesterol in the blood and has been used as an antilipidaemic drug since 1962. The active metabolite of clofibrate is clofibric acid, also known as 2-*p*-chlorophenoxyisobutyric acid (CPIB) [1]. Derivatives of fibric acid (fibrates) have been used clinically to treat dyslipidaemias; ciprofibrate and long-acting forms of gemfibrozil (fenofibrate and bezafibrate) have been developed.

Widely-prescribed fibrates in the United States include gemfibrozil and fenofibrate, whereas bezafibrate and ciprofibrate are available in other countries. Fibrates are the drugs of choice in the management of hypertriglyceridemia but the efficacy of fibric acid derivatives in both the primary and secondary prevention of atherosclerosis has remained widely in doubt [2]. Early primary prevention studies of atherosclerosis using the fibric acid derivative clofibrate showed only modest effects on atherosclerosis and an alarming increase in mortality in the intervention group [3, 4]. The use of fibrates has been associated with fatal myocardial vascular accidents, and have been reported to induce arrhythmia, which is resolved by the fourth week after treatment is stopped [5].

The benefits of lipid-lowering drug treatment for the secondary prevention of coronary heart disease have been well established by randomized, controlled trials [6]. Lipid-lowering therapy reduces stroke incidence in coronary patients, especially when total cholesterol level is lowered to less than 232 mg/dL (6.0 mmol/L) [7]. Studies have shown that fibrates decreased coronary heart disease (CHD) mortality by 13% but increased non-CHD mortality by 30%. These observations suggest that the decrease in cholesterol is beneficial, but that fibric acid derivatives increase the risk of mortality in non-coronary heart disease. Cholesterol lowering confers an overall benefit by the reductions in CHD and totally mortality. Thus, patients on certain fibrates treatment, specifically clofibrate, appear to have an increased risk of non-CHD and higher total mortality [8].

These observations imply that fibric acid derivatives may cause profound changes in myocardiocyte structure and function.

The ability of eukaryotic cells to adopt a great variety of forms and carry out coordinated movements depends on a complex mesh of filamentous proteins present in the cytoplasm known as the cytoskeleton. The cytoskeleton has a dynamic structure that also is responsible for cell division and the cell's response to external stimuli. Cytoskeletal function depends on three types of filamentous proteins such as actin, microtubules and intermediate filaments such as vimentin and desmin. Intermediate filaments (IF) containing vimentin are the most widely distributed. Desmin IFs are found predominantly in skeletal, cardiac, and smooth muscle cells. Vimentin is a 57 kDa protein which interconnects the nuclear and plasma membranes, maintaining the position of the nucleus within the cell. Desmin is 53 kDa, and both types of filaments are able to copolymerize in order to form intermediate filaments that contain both types of subunits. IFs provide mechanical strength and serve to maintain the cellular structure [9].

There are numerous experimental studies on the role of cytoskeletal alterations (especially of microtubules and desmin) in cardiac hypertrophy and failure (CHF). It has been established that heart failure has a morphological basis, i.e. myocyte degeneration resulting in cellular atrophy and interstitial fibrosis, which represent structural correlates of reduced ventricular function [10]. In studies using desmin knockout mice, desmin was found to be essential for myofibrillar functional integrity and the maintenance of general cellular integrity, e.g. for the position of the nucleus [11]. In mice with desmin null mutations, degeneration of cardiac muscle was observed indicating a role of desmin in cell survival and sar-comerogenesis [12].

Several myopathies and cardiomyopathies have been characterized by the presence of altered intermediate filaments. The loss of desmin filament function in cardiac and skeletal muscle myocytes would be expected to affect not only mitochondrial function [13] but also active force generation [14] in the cardiomyocytes [15]. The cytoskeleton is not only involved in cellular stability and integrity but plays a significant role in transmitting signals from the cellular membrane to the nucleus. Integrins can act as mechanoreceptors, aiding in the transfer of force through the cytoskeleton [16].

We have previously reported changes in the protein profile of erythrocyte cell membranes of Sprague Dawley male rats treated with clofibrate in daily doses of 300 mg/kg for 40 days. We observed an increase in β -spectrin, a decrease in ankyrin, and a decrease of the protein bands 4.1 and 6. Also, alterations in erythrocyte morphology and a decrease in osmotic resistance were observed [17, 18], suggesting that these proteins may have an important role in the structural and functional properties of erythrocytes. Consequently, it is

hypothesized that clofibrate may also modify the cytoskeletal proteins in other types of cells (particularly myocytes), as fibric acid derivatives cause profound changes in myocytes structure and function. Also, the exposure of myocytes to clofibric acid *in vitro* induced a depletion of vimentin (26.3%) and desmin (42.1%) [19]. It is clear that we need to reconsider the poorly-characterized role of fibric acids at the cellular level.

This experimental study was conducted to investigate the *in vivo* effects of clofibrate and clofibric acid on desmin and vimentin content in rat myocytes.

Materials and Methods

Reagents. Clofibric acid, clofibrate, propylene glycol, NaCl, Tris-Cl, CaCl₂, ATP, 2-Mercaptoethanol, Triton X-100, bovine serum albumin, Coomassie brilliant blue R-250, anti-desmin or anti-vimentin monoclonal antibodies, Tween 20, alkaline phosphatase (AP)-conjugated anti-mouse IgG, 5-bromo-4-chloro-3-indolyl phosphate (BCIP, AP substrate), nitro-blue tetrazolium (NBT), tetrazolium salts, EDTA, MnCl₂, formaldehyde and FITC-conjugated anti-mouse IgG, were all obtained from Sigma Chemical CO (St Louis, MO USA). Bradford Protein Assay was purchased from Bio-Rad, Laboratories (Hercules, CA USA), sodium pentobarbital (Anestestal) from Pfizer (Mexico) and from Difco (Detroit, MI USA). Methanol and acetic acid were obtained from J.T. Baker (Mexico). All other reagents were analytical reagent grade or of the highest quality available.

In vivo experiments. There is a wide variability in the doses used in several studies, but all of them used a dose lower to the LD₅₀ (p.o. is 700mg/kg in this species) (19). According to previous studies in our lab we have determined that a dose of 300 mg/kg is enough to modify the erythrocyte membrane protein profile [18].

The experiments were carried out using four-week-old Sprague Dawley rats which were acclimatized for one week prior to experimentation. Animals were fed with Purina rodent laboratory chow 5001 PMI and water *ad libitum* and were maintained in an environmentally controlled room at 25 ± 2°C and 50-70% humidity with a 12/12 h light/dark cycle throughout the experiment. The control group (n=5) received propylene glycol (control), one treatment group (n=5) received 300 mg clofibric acid /kg body weight/day and the other treatment group (n=5) received 300 mg clofibrate /kg body weight/day by esophageal gavage. All animals received the drug vehicle (propylene glycol, 2.0 ml /kg/ day).

After 30 days, the animals were sacrificed with an intraperitoneal 50 mg/kg dose of sodium pentobarbital and the hearts were excised.

Preparation of homogenate for SDS-PAGE. Each heart was processed independently. Hearts were rinsed in 10 ml 40 mM NaCl to eliminate excess blood, minced and homogenized at 4°C with a Potter-Elvehjem Tissue Homogenizer for 30 sec at 60 strokes/min. The suspension was spun at 13,000xg for 5 min and the pellet resuspended in 10 ml of buffer A (2 mM Tris-Cl, 0.2 mM CaCl₂, 0.2 mM ATP, 0.2 mM 2- Mercaptoethanol, 0.1% Triton X-100 pH 8). The pellets were washed in 10 ml of buffer A. Protein content was determined by the method of Bradford [20] using bovine serum albumin as standards. Homogenates were used immediately or stored at -70°C. Proteins were resolved with SDS-PAGE [21] and stained with Coomassie brilliant blue R-250.

Western blotting analysis. The proteins were separated using reducing SDS-PAGE (10%) and electrophoretically transferred to unmodified nitrocellulose (0.45 μ m) according to Burnette [22]. The membranes were blocked with 10% (w/v) non fat dry milk in TBS (20 mM Tris-Cl, 0.9% NaCl, pH 7.5) at 25°C for 24 h. Membranes were probed with anti-serum, anti-desmin or anti-vimentin monoclonal antibodies at a titer of 1:200 for 2 h at room temperature. Following three washes with TTBS (0.5% Tween 20 in TBS, pH 7.5), the membranes were incubated with the secondary antibody, anti-mouse IgG-AP conjugate (1:15,000). The blots were developed with BCIP. The relative amounts of desmin or vimentin detected on Western blots were quantified by scanning densitometry (Appraise Junior Densitometer, Beckman).

Densitometric analysis. Gels were photographed with plastic film and the bands were analyzed by densitometry (Appraise Junior Densitometer, Beckman) at a wavelength of 540 nm.

Statistical analysis. One-way ANOVA with the Newman-Keuls test for multiple comparisons at the interpopulation level was used to investigate any possible difference between the relative percentage of the protein bands in rats treated with clofibrate, treated with clofibric acid and untreated.

Results

Effect of clofibrate or clofibrinic acid on myocytes in vivo. In our *in vivo* model system, we were able to observe alterations in the content of vimentin and desmin in samples obtained from animals treated with clofibrate or clofibrinic acid. When the proteins of the cytoskeleton were analysed by SDS-PAGE, we observed that clofibrinic acid caused a reduction in the density of bands corresponding to the molecular weights of desmin and vimentin (Fig. 1). Clofibrate did not cause any apparent changes. However, when the proteins were analysed by immunoblotting, we observed a significant decrease in cardiac vimentin and desmin of animals treated with either clofibrate or clofibrinic acid (Fig. 2 A, B).

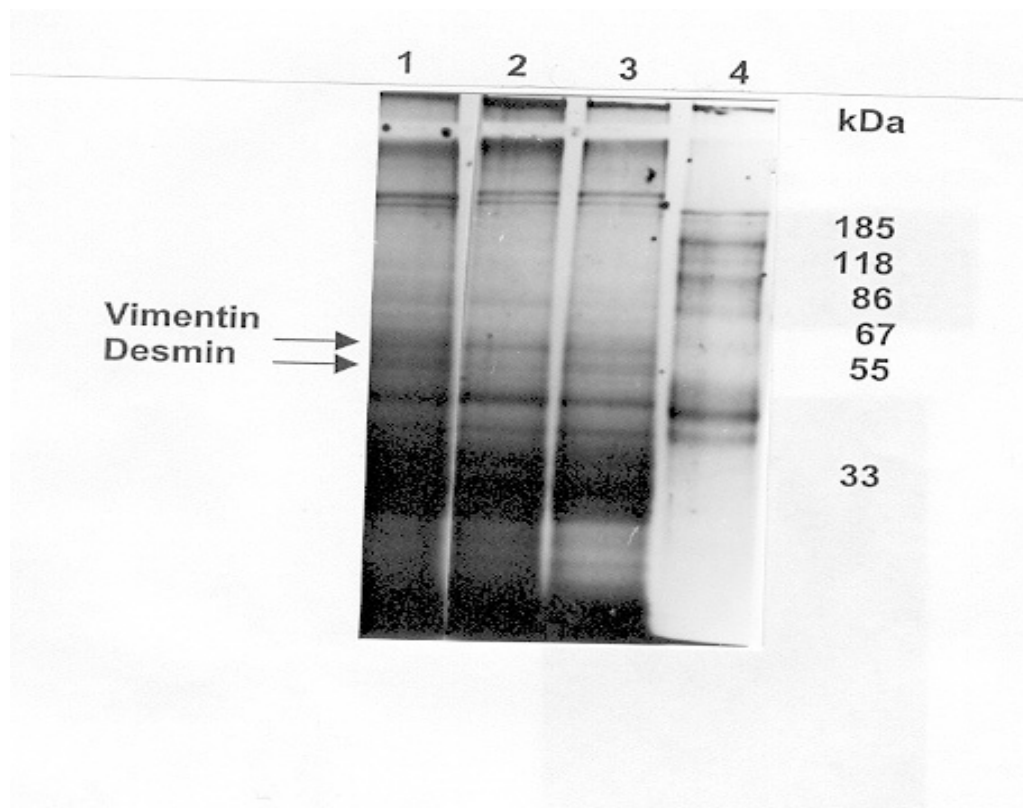


Figure 1. Effect of clofibrate or clofibrinic acid (300 mg/kg body weight administrated to rats, daily for 30 days) on vimentin and desmin concentration in myocytes . Proteins (25 μ g) were separated by SDS-PAGE and analyzed by densitometry.

A. Lane 1 corresponds to the extract of untreated cells, lane 2 to the extract of cells treated with clofibrinic acid, and lane 3 to the extract of cells from rats treated with clofibrate. Molecular weight markers are shown in lane 4. The expected molecular weights of bands corresponding to vimentin and desmin are indicated with arrows.

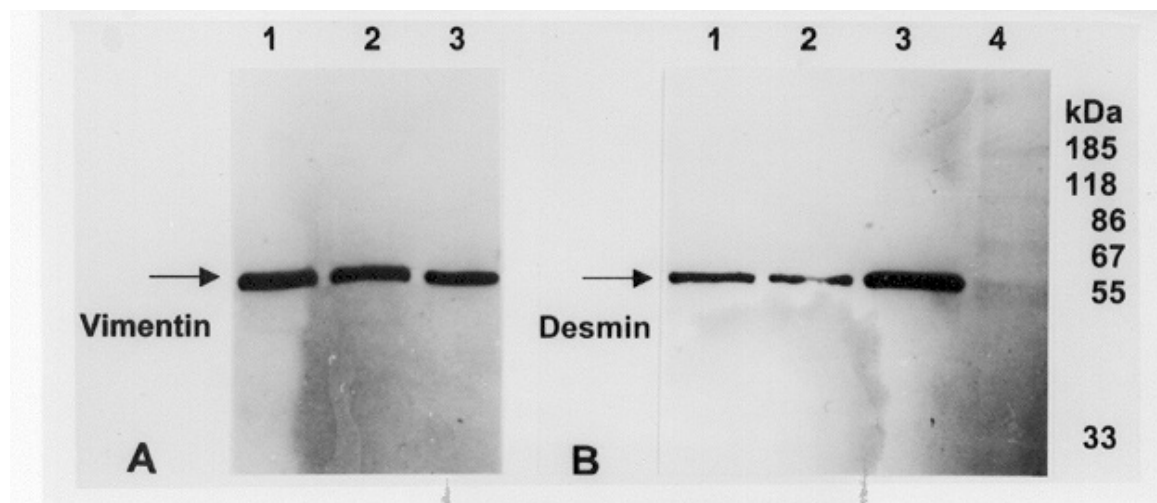


Figure 2. Western Blot analyses of vimentin and desmin. SDS-PAGE gels were transferred to nitrocellulose membranes and the cytoskeletal proteins detected with monoclonal antibodies. Each lane contains 25 μ g protein.

A. Anti-vimentin: Lane 1, 25 μ g myocardiocyte extract from rats receiving no treatment; lane 2, 25 μ g myocardiocyte extract from rats receiving clofibric acid (300 mg/kg/day); lane 3, 25 μ g myocardiocyte extract from rats receiving clofibrate (300 mg/kg/day).

B. Anti-desmin: Lane 1, 25 μ g myocardiocyte extract from rats receiving clofibrate (300 mg/kg/day); lane 2, 25 μ g myocardiocyte extract from rats receiving clofibric acid 300 mg/kg/day); lane 3, 25 μ g myocardiocyte extract from rats receiving no treatment. Molecular weight markers are shown in lane 4.

Table 1 shows the peak areas of densitometric scans of the SDS-PAGE gels. These data show that with clofibric acid treatment, there were significant decreases in both vimentin and desmin (11.7% and 31.9%, respectively) compared to control in heart harvested from the rats. Clofibrate values were not significantly different compared to untreated rats.

Table 2 shows the areas under the curves of densitometric scans of Western blots. Immunodetection reveals that both clofibric acid and clofibrate treatments caused a significant decrease in the vimentin and desmin content. Clofibric acid treatment showed decreased amounts of vimentin (27.4%) and desmin (49%), and with clofibrate the amount of vimentin was 29.5% and desmin was 48.7% of the control.

Table 1

Effect of treatment with clofibric acid and clofibrate on the amount of cytoskeletal proteins in myocardiocytes from rats.

Relative percentage of the protein bands.

Area under the curve (mm²)

	Untreated (n=5)	clofibric acid (n=5)	clofibrate (n=5)
Vimentin	100% (4.57±0.58)	88.3% (2.53±0.29)*	92.7% (4.24±0.41)
Desmin	100% (2.54±0.32)	68.1% (1.73±0.10)*	95.5% (2.42±0.30)

Values in parentheses represent the area under peaks of absorbance at 540 nm, of protein bands on SDS.

The values are presented as mean ± SD

n = 5 separate experiments.

*Different to untreated, *p* < 0.05.

Table 2

Effect of clofibric acid and clofibrate on the cytoskeletal proteins concentration, vimentin and desmin.

Relative percentage of the protein bands.

Area under the curve (mm²)

	Untreated (n=5)	clofibric acid (n=5)	clofibrate (n=5)
Vimentin	100% (7.46±0.71)	72.6% (5.42±0.26)*	70.5% (5.26±0.21)*
Desmin	100% (6.98±0.32)	51.0% (3.56±0.07)*	51.3% (3.58±0.07)*

Values in parentheses represent the area under peaks of absorbance at 540 nm, of protein bands detected with anti-vimentin or anti-desmin on Western blots.

The values are presented as mean ± SD.

n = 5 separate experiments.

*Different to untreated, $p < 0.05$.

Discussion

The fibrates available in the United States are clofibrate, gemfibrozil, and fenofibrate. Clofibrate is usually administered at 1000 mg BID (twice a day), gemfibrozil at 600 mg BID, and fenofibrate at 200 mg/d in a single dose. Other fibrates available in other countries are bezafibrate and ciprofibrate. Prescriptions for medications that diminish the concentration of serum lipids have increased greater than tenfold, totalling in the United States more than 26 million in 1992 [23] and over 100 million worldwide [24]. Previous studies using the fibric acid derivative, clofibrate, showed only modest effects on atherosclerosis and an alarming increase in mortality in the intervention group [3].

We examined the effect of clofibrate and clofibric acid on the fundamental cytoskeletal proteins in myocardiocytes *in vivo*. On the basis of previous studies [17, 18] we suspected that a possible explanation for the undesirable cardiac effects could be related to the effects of fibric acid on cardiac cell proteins.

The dose of clofibrate was within the range used in most studies in rats. In comparison with the recommended dose in humans: (2 g/day, approximately 30 mg/kg in a 70 kg person), we administrated 10 times as much, but, due to species differences, rats are less sensitive than humans to clofibrate (IARC) [25]. The decreases in Vimentin and Desmin levels suggest a direct alteration of the proteins or their synthesis, as has been previously described for the cytoskeletal proteins of rat erythrocytes treated with clofibrate [17].

In vitro studies showed decreases of vimentin and desmin in chicken myocardiocytes exposed to fenofibrate [5]. Using others fibric acid derivatives, those authors also observed 12% and 19% increases of vimentin and desmin, respectively, in the cytoplasmic fraction of chicken myocardiocytes treated with gemfibrozil. Bezafibrate had no effect on vimentin content but caused a 17% increase in desmin compared to control [5].

The effect of the clofibric acid on the cytoskeleton may be due to an alteration of the structure of vimentin and desmin. Vimentin may participate in the mechanical support of the nucleus and the maintenance of its position within the cell. Desmin filaments of muscle cells organize the array of microtubules [15]. Also, the organization of desmin provides evidence for its supportive function in the maintenance of structural integrity and function of cardiac muscle cells [26].

Previous work has been conducted to determine structural and functional defects in a pathogenic desmin variant. Those authors identified a novel heterozygous Q389P desmin mutation and showed that the mutant is incapable of constructing a functional intermediate filament network and has a dominant negative effect on filament formation. These results show that desmin plays an essential structural role in maintaining muscle integrity [27]. In transgenic mice lacking desmin, for example, this supporting architecture is disrupted and muscles are misaligned [14]. Also, some studies found changes in myocardial cytoskeletal intermediate filaments and myocyte contractile dysfunction in dilated cardiomyopathy in humans, showing alterations of cytoskeletal intermediate filament distribution [28].

The present work shows that under the experimental conditions described, clofibrate and fibric acid cause a decrease in the quantity of vimentin and desmin and an alteration of the normal structure of the cytoskeleton of myocytes, *in vivo*. Considering the role that intermediate filaments play in the dynamic structure of the cytoskeleton, it is likely that an alteration in the homeostasis of vimentin and desmin will directly effect the cytoskeletal organization of myocytes. This likely contributes to the mechanism of their toxic action.

Conclusion

Our results show that the concentration of vimentin and desmin in rat myocytes diminishes significantly after treatment with clofibric acid. Intermediate filaments are present only in cells that display multicellular organization. One essential role of intermediate filaments is to distribute tensile forces across cells in a tissue, anchoring intracellular arrays to the plasma membrane. Since IFs are actively involved in the muscular contraction, we propose that alterations in the cytoskeleton could affect the functionality of heart fibres. In our previous study, we have found changes in the erythrocytic membrane protein profile caused by fenofibrate, bezafibrate and gemfibrozil (unpublished data). We suggest that fibric acids are able to modify the organization and the content of cytoskeletal proteins in rat myocytes and that these alterations may be related to the adverse secondary effects.

The extracellular matrix controls cytoskeletal mechanics and structure, and an alteration of proteins would establish a causative link between the amount of extracellular matrix proteins and the changes of the cytoskeleton in cardiac failure. Therapeutic approaches should take into account alterations of the cytoskeleton in the development of end-stage heart failure. Clofibrate, which we have shown to cause such alterations, needs to be evaluated in this context.

Acknowledgements

We gratefully acknowledge the excellent technical assistance of Dr. Antonio Luna de la Rosa

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